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The Lewis Y antigen is a breast cancer associated carbohydrate antigen. The basis of the current program is to utilize structural information for Lewis Y-antibody interactions to develop novel immunotherapeutics for breast cancer treatment. It is postulated that the Lewis Y determinant on human breast adenocarcinoma cells is of key importance since it mediates internalization and lethal function of Lewis Y specific MAb. To develop such immunotherapeutics, we have established the molecular recognition properties of several anti-Lewis antibodies for Lewis Y, we have established that peptide mimotopes of adenocarcinoma associated carbohydrate forms that include Lewis Y may function as a breast cancer vaccine, and have established that a molecular basis for such mimicry.

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FOREWORD

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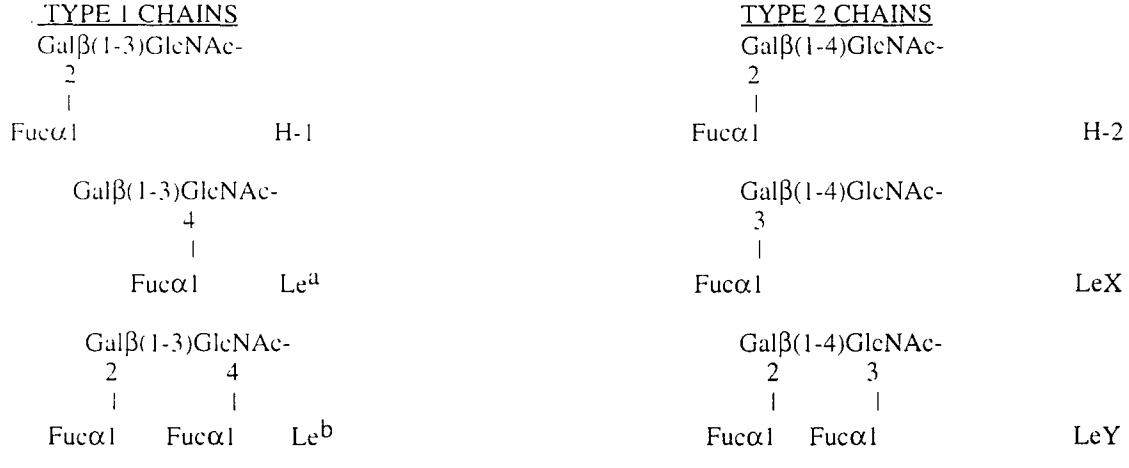
Introduction

Lewis antigens are blood group carbohydrate antigens implicated as potential target antigens in a number of cancers (1). The expression of $\alpha 1 \rightarrow 2$ fucosylated structures such as Lewis Y (LeY), H-2 and Lewis b (Leb) (fig 1) is inversely correlated with the survival of patients with primary lung adenocarcinoma, suggesting that these determinants promote invasiveness (2). The difucosylated neolactoseries structure Lewis Y, Fucα1$\rightarrow$2Galβ1$\rightarrow$4(Fucα1$\rightarrow$3)GlcNAcβ1$\rightarrow$R, associated with several human tumors such as breast, lung and gastrointestinal carcinomas is specifically detected by MAbs. The expression of LeY blood group antigen in epithelial cancer tissues and cell lines has been studied using LeY-specific monoclonal antibodies (2-6). Using immunochemical approaches, LeY epitopes are found to be expressed on MUC-1 mucins, lower m.w. glycoproteins and glycolipids, as well as higher m.w. proteins like CEA and LAMP-1 (3, 4, 7, 8). The presence of LeY epitopes on a number of different molecular carriers, explains the high incidence of LeY associated with breast cancer. The high expression of LeY in breast and other human cancers of epithelial origin and the availability of specific murine and humanized MAbs make LeY an attractive candidate target for clinical studies.

The blood group-related neolactoseries carbohydrate structures Lewis X (LeX), sialyl-LeX (sLeX), ABH, Lewis a (Lea), sialyl-Lea (sLea) and LeY are examples of terminal carbohydrate structures related to tumor prognosis (1, 2). The core components of Le antigens are structurally very similar (Figure 1). These antigens constitute carbohydrate moieties of tumor associated gangliosides, the human carcinoembryonic antigen family, the human pancreatic MUC-1 antigen and are identified in carcinomas of the skin, stomach, pancreas, lung, colon, breast and prostate. The histo-blood group related antigens sLeX and sLea, are also implicated as immunogenic antigens in human melanoma (9). Melanoma patients immunized with a MCV expressing these antigens developed high titers of IgM but not IgG to both ligands. IgM titers in normal subjects were found to be low. It is noteworthy that patients thus far who developed high titers of anti-sLe antigen IgM showed no evidence of hematologic toxicity (hemolysis, anuria or granulocytopenia) (9), despite the notion that these antigen types are displayed ubiquitously.

Figure 1



Structures of H, Lea, Leb, LeX and LeY blood group determinants. Fuc: L-fucose; Gal: D-galactose; GlcNAc: N-acetylglucosamine. These oligosaccharides are found at the non-reducing termini of sugar chains in glycolipids, glycoproteins and mucins.

Three important criteria suggest that lactoseries structures are potential targets for immunotherapy in humans: (1) their specific up-regulation (density of expression) on tumor cells; (2) their function as differentiation antigens; and (3) their role in cell adhesion and motility underlying their metastatic potential. The expression of LeX, LeY and sLeX in neutrophils is limited to humans (10). Therefore, immune responses against such carbohydrates are expected to be weaker in humans compared to other mammals. Tumor associated carbohydrate antigens (including LeY) are expressed at low levels on normal tissues. While LeY structures have not been chemically isolated from neutrophils, it is possible neutrophils do express low levels of

LeY, while the expression of extended LeY with internally fucosylated structure (LeY-LeX) is limited in normal cells and tissues.

Several MAbs generated against LeY have been described in the literature, although they differ in the recognition of specific epitopes. Two antibodies called BR55-2 and 15-6A have been previously described by Dr. Steplewski (co investigator on this project) and colleagues, that bind to the LeY antigen on breast carcinoma cells (3, 11, 12). The monoclonal BR55-2 (IgG3) in particular, and its isotype switch variants directed against the LeY oligosaccharide are found to mediate ADCC (antibody-dependent cell mediated cytotoxicity) with human and murine effector cells, its IgG3 and IgG2a isotypes are highly active in CDC (complement-dependent cytotoxicity) and both efficiently inhibit tumor growth in xenografted nude mice (11-13). The MAb BR55-2 was one of the first anti-LeY antibodies shown to mediate ADCC with human and murine effector cells. A limited pilot study in breast cancer patients using BR55-2 (IgG3) indicates the therapeutic potential of BR55-2 in minimal residual disease (14).

The basis of the current program is to utilize structural information for Lewis Y-antibody interactions, particularly for BR55-2, to develop novel immunotherapeutics for breast cancer treatment. It is postulated that the LeY determinant on human breast adenocarcinoma cells is of key importance since it mediates internalization and lethal function of LeY specific MAb. Molecular probes based on structural information and newly developed MAbs or fragments can be applied for future diagnosis in tumor progression and micrometastasis as well as immunotherapy. In addition, it appears that anti-idiotypic antibodies that mimic the Lewis Y carbohydrate antigen represent potential surrogate immunogens for specific immunization for the treatment of breast cancer. During the current funding period, we have examined the molecular basis of recognition of anti-LeY antibodies for the LeY antigen using molecular modeling, have examined how a Lewis Y mimicking peptide can bind to BR55-2 (and to another anti-LeY antibody B3) and we have examined the extent peptides that mimic carbohydrate subunits can induce humoral responses in mice that can target breast tumor cells in vitro and in vivo.

Body

We have published 6 papers in the last 2 years (15-20) with 1 more in press, 1 submitted and several more in preparation that are relevant to the proposed studies in this application. It is impossible to show all the data. Consequently, the following summaries the most salient results of our present funding period.

Molecular recognition of LeY

A lot is now known about the structure/function relationships of anti-LeY antibodies. Defining the configuration of native Lewis structures recognized by antibodies is important for understanding the basis for antigen specificity. In our last funding period (previous progress report) we defined how BR55-2 binds to LeY (18). Our modeling study showed that BR55-2 shares similar recognition features for the difucosylated type 2 lactoseries LeY structure observed in the crystal structure of another anti-LeY antibody BR96, co-crystallized with a nonoate methyl ester LeY tetrasaccharide (21). We observed that a major source of specificity for the LeY structure by anti-LeY antibodies emanates from interaction with the β-D-N-acetyl-glucosamine (GlcNac) residue and the nature of the structures extended at the reducing site of the fucosylated lactosamine. We have recently shown that the nature of LeY binding is extend to the anti-LeY antibody B3 (manuscript #1 in appendix). Molecular modeling of B3 complexed with the putative tetrasaccharide core of LeY was performed based upon the BR96-sugar recognition scheme as in our BR55-2 study. The B3 model emphasizes key polar and nonpolar interactions contributing to the molecular recognition feature for Le-Y shared among related anti-LeY antibodies, and consistent with epitope mapping profiles of lactoseries derivatives reactive with B3 (8). The relationships among B3, BR96 and BR55-2 further allows for mutational analysis to be performed on BR55-2. Mutational studies and the generation of single chain Fvs (scFvs) of anti-LeY forms provides an enormous amount of information correlating structure/function relationship (22-27). Many mutations have been suggested for BR96 to improve its antigen affinity. One of which is a conversion of an Asp residue at position 96 to an Ala residue. Asp is found at this position in BR55-2 as well, but B3 contains an Ala. We have found that substitution of Ala for Asp in BR55-2 increases the intermolecular interaction energy of BR55-2 for the LeY tetrasaccharide core by as much as 20Kcal/mole (18). We are now evaluating the various mutations around the LeY binding site to establish the extent to which changes will increase the affinity of BR55-2 for LeY.

Expression of scFv fragment of hu-BR 55 -2 mimic: We have made and expressed a single chain Fv fragment of humanized BR55-2. The scFv has been initially cloned into the vector pCANTAB 5E and

expressed in the E. Coli strain HB2151 (Pharmacia) and in Phage expressed in TG1 cells (Pharmacia). To express the protein in H2151 cells, transformed cells are cultured overnight growing in glucose containing media at 28C then changed to glucose-free media with 1 mM IPTG and continued to incubate at the same condition for another 20hrs. The cells are pelleted and sonicated on ice. Both culture supernatant and pellet lysate supernatant were tested using ELISA. Our previous result showed that this scFv fragment is not secretable and mostly formed as an inclusion body, which is solubilized in 6M guanidine hydrochloride pH 7.0 and then tested by ELISA as well. Using information on enhanced affinities for mutated BR96 and the various anti-LeY antibody sequences available in the GenBank database we are using saturation mutagenesis approaches and site-specific mutagenesis approaches to construct mutated scFv forms. Mutated scFv forms expressed on Phage allows for repeated panning against LeY expressing breast tumor cells to identify high affinity binding scFv. Isolated scFvs will be sequenced and molecular modeling will be used to establish the source of LeY specificity. A judiciously chosen scFv will be used for further studies as described in our original application. These will include the generation of multimeric forms to increase valency and the construction of bispecific forms using the anti-epidermal growth factor receptor antibody 425. We have previously shown in last years progress report that a covalently linked bispecific BR55-2 and 425 form displays enhanced binding characteristics to LeY expressing tumor cell lines.

In vitro and in vivo functionality of Le antigen mimicking peptides

Very few groups are investigating carbohydrate based vaccines or carbohydrate based immunotherapy. One major reason for this is that carbohydrate antigens are expensive and very difficult to synthesize. Further, expression of tumor-associated carbohydrate antigens is by no means specific to tumors. Crucial issues are expression of antigen density, multivalency, reactivity threshold of antibody binding, and efficient production of antibody having a strong complement-dependent or T cell dependent cytotoxic effect on tumor cells without damage to normal tissues. Studies on cancer vaccine development depend on many factors for success that include: 1) Selection of carbohydrate epitopes; 2) Design and assembly of epitopes coupled to macromolecular complex as an efficient immunogen: 3) establishment or availability of a good animal model; 4) Evaluation of immune response in animals; tumor rejection without damage to normal tissues; and 5) careful clinical application. Since carbohydrate antigens are generally weakly immunogenic in humans, only short lived IgM responses have been historically observed. The importance of adjuvant sublimation is highlighted in such studies to offset the relatively weak immunogenicity of the carbohydrate structures. In addition, antibodies to carbohydrates are typically of low affinity and the notion of how cellular immunity is modulated by carbohydrates is unclear.

While a LeY-conjugate vaccine is attractive for development, antibodies generated against synthetic LeY are not always cross-reactive with native LeY antigen forms (28). This general phenomenon has also been observed with sialyl-Tn (sTn) formulations, suggesting that neoglycoproteins containing sTn in which the carbohydrate structures are clustered together would make better immunogens (29, 30). This has also been observed with GM2-KLH formulations in which the majority of IgG antibodies induced by the GM2-KLH/QS-21 vaccine while reactive in ELISA, failed to react with cell surface expressed GM2. So while synthetic LeY formulations induce anti-synthetic LeY reactivities, the generated antibodies may not bind to tumor cells or bind very weakly. This observation formulates one of the rationales for our studies. In our original application we anticipated developing anti-idiotypes to BR55-2. We have since changed this view to examine peptides derived from random peptide phage libraries. The notion of using peptide mimics of carbohydrates to induce anti-carbohydrate immune responses parallels the use of anti-idiotypic antibodies as immunogens.

It is a reasonable hypothesis that diminished anti-carbohydrate immune responses observed in clinical trials might be due to the development of tolerance. Data exist showing that an acquired state of tolerance to one antigen form can be broken by using a different molecular form of the same antigenic moiety. This could be an important consideration in a broader context such as in the immunotherapy of cancer patients, who are often times immunodeficient or tolerant against their own tumor. For these antigen types the idea of using surrogate antigens that mimic carbohydrate antigens might be a viable approach to break an inherent inability to stimulate T cell immune responses against T independent antigens associated with tumors. A peptide mimic might be able to break such tolerance. Mimicking peptides represent a new and very promising tool to overcome T cell-independence and to increase the efficiency of the immune response to carbohydrates. In addition, carbohydrates expressed on the surface of tumor cells might be clustered epitopes, making it even more difficult to synthesize them properly. Mimicking peptides synthesized as clusters might therefore be

superior immunogens, eliciting responses that cross-react with native carbohydrate conformations. The notion of using peptide mimics to induce anti-tumor immune responses parallel the use of anti-idiotypic (ant-Ids) antibodies that mimic carbohydrates as immunogens. The immunomodulatory activities of anti-idiotypic Abs both in animals and in patients have been demonstrated (31). Cancer patients administered anti-carbohydrate antibodies that have high anti-idiotypic antibody levels, display higher survival rates compared to those where low or no serum anti-idiotype levels were detected (32-34). Anti-idotypic antibodies mimicking tumor antigens induce anti-anti-idiotypic proliferative T lymphocytes of the helper and suppressor type, as well as cytotoxic lymphocytes (31). These results point to beneficial anti-tumor in vivo effects associated with antibody-mediated tumor targeting. Peptides that mimic carbohydrate structure have significant advantages as vaccines compared with carbohydrate-protein conjugates or antiidiotypic antibodies. First, the chemical composition and purity of synthesized peptides can be precisely defined. Second, the immunogenicity of the peptides can be significantly enhanced by polymerization or addition of relatively small carrier molecules that reduce the total amount of antigen required for immunization. Third, peptide synthesis may be more practical than synthesis of carbohydrate-protein conjugates or the production of anti-idiotypes. Fourth, peptide mimicking sequences can be engineered into DNA plasmids for DNA vaccination to further manipulate T cell responses.

The basic hypothesis of this application is that peptide mimotopes can induce functionally relevant immune responses **in in vivo and in vitro models.** Aromatic-aromatic interactions appear to mimic a variety of carbohydrate subunit interactions (Table 1). We have shown that peptides containing such motifs induce sera

Table 1. Peptide motifs that mimic carbohydrate structures

Motif	Carbohydrate	Structure
YYPY	Mannose	methyl-α-D-mannopyranoside
WRY	Glucose	α(1-4)glucose
PWLY	Lewis Y	Fucα1→2Galβ1→4(Fucα1→3)GlcNAc
YYRYD	GroupC Polysaccharide	α(2-9)sialic acid

that is highly functional both in vitro and in vivo. We showed in vivo protective immune responses in peptide immunized mice against a lethal challenge of Neisseria meningitidis (35). The immunizing peptide (peptide-proteosome conjugates) mimics the major group C meningococcal polysaccharide (MCP) (35). We have recently reported that peptides containing aromatic motifs (Table 1) can effectively mimic mannose, sialyl and histo-blood group related carbohydrate epitopes (particularly LeY) expressed on breast tumor lines (36) (manuscript #2 in appendix) and found on the major envelope protein of the human immunodeficiency virus (HIV) (17) (Manuscript #3 in appendix). The basis for these studies was our observation that the LeY tetrasaccharide structure is similar to the core structure of MCP, suggesting that it is possible for antibodies to cross-react with these two moieties. In the former studies we have found that carbohydrate-mimicking peptides retain carbohydrate-like conformations, inducing anti-carbohydrate immune responses against breast tumor cells and mediating their killing by a complement-dependent mechanism. Anti-LeY antibodies have been previously shown to neutralize HIV-1 infection in vitro (37). Consequently, we asked whether sera reactive with LeY expressing cells will also react with the envelope protein of HIV-1. The **in vitro** neutralization of HIV-1 infection of target cells was specific and similar to human sera from infected subjects. The neutralization pattern could be modified by changing one amino acid in the immunizing peptide (17).

Further evidence for **in vivo** functionality of peptide mimetic vaccination comes from our recent studies (manuscript in preparation) in tumor challenged mice. LeY is not expressed in mice. Subsequently a mouse model is not available to study the in vivo functionality of mice primed with peptides and then challenged with LeY expressing tumor. While rats supposedly express LeY, rat tumor lines obtained to date from ATCC, and other investigators, proved negative for LeY expression (e.g., binding to the anti-LeY monoclonal antibody BR55-2 by FACS analysis). However, mice do express sLeX. An anti-Id made against the monoclonal antibody FH-6 has proved to be an effective mimic for sLeX, increasing the median mouse survival time of anti-Id immunized Balb/c mice after tumor challenge with fibrosarcoma Meth-A tumor cells (38). A peptide with the sequence ISDGTTYTYYPDS derived from CDR2 of the heavy chain of the anti-Id appears to be

responsible for a portion of this anti-tumor response (38). This anti-Id derived peptide displays homology with our aromatic peptides in being composed of Tyr residues and displaying homologous hydroxyl groups on the Thr residues. While we have not optimized any peptide for sLeX, we used FH-6 to screen a 15mer peptide phage library to isolate peptides. We examined one peptide having a similar sequence to that of the anti-Id peptide; GSSFWRYTTYYDPS (P4 in Table 2). Subsequently, we asked if this peptide would produce a significant anti-tumor effect in Balb/c mice in in vivo experiments.

Table 2. Peptides used in various studies presented in this application.

Peptide name	Sequence	Source
G1	GVVWRYTAPVHLGDG	ME361 phage screen
G2	LDVVLAWRDGLSGAS	ME361 phage screen
P1	GGIYYPYDIYYPYDIYYPYD	Repeating motif of Con A phage screen
P2	GGIYWRYDIYWRYDIYWRYD	Repeating motif from amylase inhibitor
P3	GGIYYRYDIYYRYDIYYRYD	Repeating motif of anti-Id
P4	GSSFWRYTTYYDPS	FH-6 phage screen
B1	IMILLIFSLLWFGGA	BR55-2 phage screen
C1	GDTRYIPALQHGDKK	Irrelevant Control

We first verified sLeX expression on Meth-A tumor cells ($H2^d$) using the anti-sLeX antibody FH-6 by FACS analysis using our previously defined protocols (data not shown). We then verified that the P4 peptide antigenically and immunologically mimicked sLeX by a series of experiments (manuscript in preparation). We verified that the P4 peptide blocked FH-6 to Meth-A cells and competed with a synthetic sLeX probe (GlycoTech Inc) for binding to FH-6. We verified that sera raised against a P4-proteosome formulation binds to Meth-A by FACS and binds to synthetic sLeX by ELISA. These data verified that the P4 peptide was mimicking sLeX both antigenically and immunologically. We next examined the survival of groups of host mice preimmunized with the P4-proteosome conjugate compared with control immunizations that included peptide mimics of LeY, followed by challenge with sLeX expressing Meth-A cells (Table 3). In these experiments groups of Balb/c mice were immunized i.p. with 100ug of either P1, P2, P3, P4, G1 and C1 coupled to proteosomes (Table 2), three times at 2-wk intervals. Separate groups were immunized with control carrier protein or adjuvant. Ten days after the third immunization, mice were challenged subcutaniously (sc.) with 10^6 live Meth-A cells expressing sLeX antigen (day 0). Survival times of host mice were monitored. These results (Table 3) indicate that preimmunization with a sLeX mimicking peptide induced an immune response that prolonged survival time. The p value was <0.001 for the P4 peptide when the generalized Wilcoxon test was performed on the differences between the survival of the group preimmunized with peptide and that of the group preimmunized with a control peptide, or that of the group receiving no treatment.

Table 3. Summary of results of tumor challenge

Immunogen	Nominal antigen mimicked	Number of mice immunized	Survival Time (months)	Statistical significance compared to:	
				None	C1
P1	Le	12	2.44±1.23	p<0.05	p<0.05
P2	Le	12	1.56±0.35	N.S	N.S
P3	Le	12	2.40±1.32	p<0.05	p<0.05
P4	sLeX	12	3.60±1.56	p<0.001	p<0.005
G1	GD2/GD3	11	1.45±0.33	N.S.	N.S.
QS-21	-	11	1.51±0.43	N.S	N.S.
Proteosome	-	11	1.48±0.42	N.S.	N.S.
C1	-	11	1.44±0.22	N.S.	-
None	-	12	1.46±0.26	-	N.S.

None is tumor alone. N.S. Not Statisticaly significant.

Some protection was afford by P1, P2 nd P3 peptides (Table 3). ELISA reactivity with these sera indicated cross-reactivity with sLeX (Manuscript #4). ELISA reactivity of FH-6 with these three peptides dispayed as multiple antigen presentation (MAP) peptides indicate that these peptides mimic sLeX (Figure 2). FH6 does not react with other peptides such as 104 (referred to as B1 in Table 2). These results indicate that the motifs can induce immune responses that target a tumor associated antigen in vivo.

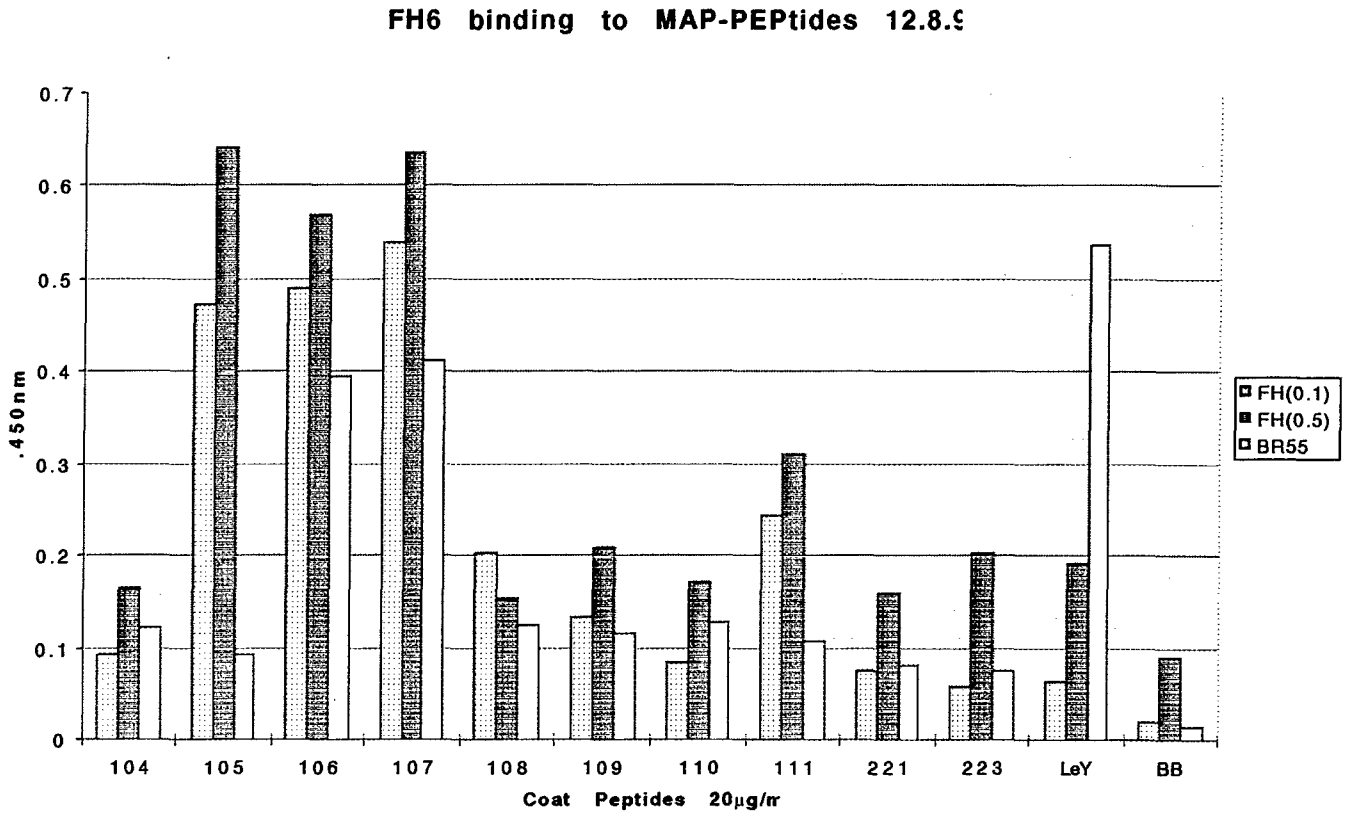


Figure 2. Reactivity of the anti-sLex antibody FH-6 with MAP peptides. 105 corresponds to P1, 106 corresponds to P2, 107 corresponds to P3, and 104 corresponds to B1 as defined in Table 2.

Specificity of anti-Peptide sera

An important consideration of using peptides as immunogens is the induction of adverse cross-reactivites with normal tissues. This is an important issue because of the perceived broad specificity of our sera for Le antigens and their constituents. It is very possible that antibodies may not cross-react with tumor cells expressing carbohydrates, while reacting with carbohydrate probes in ELISA. Published reports on polyclonal sera raised to GM2, sTn and LeY preparations, all react to these antigens in ELISA but do not bind, or bind with diminished activity, with native forms on the tumor cell surface (28-30). Consequently, reactivity with synthetic probes, broad or otherwise, does not automatically translate into sera that is capable of binding to carbohydrate expressing tumor cells or tissues. Binding to carbohydrate expressing tissues depends on the level and density of expression of the carbohydrate, and carbohydrate conformational presentation. Reactivity of a common carbohydrate epitope with different antibodies or ligands is highly dependent on the type of carrier glycosylceramide or carrier O-linked peptide (39) which can effectively restrict cross-reactivity with otherwise related carbohydrates expressed on normal tissues (or on synthetic probes). Reactivity of sera raised to di- and trisaccharides indicate restricted activity with normal tissues. Disaccharide formulations are in the clinic for TF and sTn antigens. It would seem that polyclonal sera raised to two saccharide units would lead to broad reactivity with normal tissues. This is not the case.

Immunization with carbohydrate mimicking peptides has not induced any autoimmune like symptoms nor adverse effects in mice or rabbits. These observations suggest that we are introducing minimal tissue damage in animals using the immunization schedule as described. We also saw no affect on mice hyperimmunuzied in our meningococcal studies (35). These mice received up to 100ug of immunizing peptide per week for 4 weeks. To further our studies of cross-reactivities, we have initiated screening our sera with human surgical specimens. We performed immunostaining (using an indirect immuno-peroxidase method) of tissue specimens derived from a variety of tumor types and normal tissue (Table 4 manuscript #4 in appendix) following procedures previously described (40). As a control antibody we used BR55-2. Control immunocytochemical experiments were performed by substitution of primary rabbit sera with normal rabbit serum at the same dilution. Tissues were obtained from the tissue procurement section of the Hospital of the University of Pennsylvania.

Table 4 Summary of Carbohydrate Expression on Human Tissues

		Sera Reactivity				BR55-2 reactivity			
Tissue Type	Total	+++	++	+	-	+++	++	+	-
Normal tissues									
Stomach	5			1	4				5
Pancreas	7			2	5			2	5
Ovary	20			4	16			2	18
Breast	15			4	11			2	13
Lung	4			2	2			1	3
Heart	2				2				2
Prostate	12			2	10			3	9
Thymus	6			2	4			2	4
Tumors									
Breast	20	20				19	1		
Lung	11	9	2			10	1		
Ovary	20	17	3			17	3		
Pancreas	4	2	2			4			
Bladder	5	4	1			4	1		
Prostate	6	4	2			4	2		

Carbohydrate expression was determined by the avidin-biotin-immunoperoxidase method and scored according to staining intensity and abundance of immunostaining:+++, strong, ++ moderate, + weak, - negative. The numbers under Total refer to numbers of individual samples from different individuals that were tested. The numbers in the body correspond to the number of samples that fall into specific categories of reactivity.

Briefly, rabbit polyclonal anti-sera raised against the P3 peptide-proteosome conjugate was used in this study. Rabbit sera to the P3 peptide was chosen to screen tissues because this motif peptide elicits sera that displays reactivity for MCP and LeY (17). It may be perceived that this peptide might induce sera with broad reactivity for a variety of carbohydrates expressed on the surface of human tissue. This sera is therefore seen as potentially promiscuous in binding and consequently serves as an excellent control to assess the biological relevance of the broadness in sera reactivity. Thirty-eight fresh normal tissue samples, 33 paraffin-embedded normal tissue samples, 43 paraffin-embedded epithelial tumor samples including tumors of the colon, stomach, breast, lung, prostate, bladder and pancreas and 23 fresh epithelial tumor samples were examined and graded (Table 4). These results indicate that the generated sera displays negative staining in the majority of normal samples, which is comparable to the very specific anti-LeY reactive antibody BR55-2. As expected, strong binding was observed in the majority of tumors examined. These data further suggest that the generated sera is minimally binding to normal tissues while displaying strong binding to tumor tissues that over express histo-blood group related carbohydrates. These data suggest that even though this serum reacts with common structural features of MCP and LeY, the presentation of related carbohydrate forms on tissues are affected by

the carrier molecules to which they are attached as previously suggested (39) or the density of expressed carbohydrate.

We have confirmed that the sera is reacting with carbohydrate. Anti-peptide reactive sera to LeY is inhibitable by LeY and by specific peptides, and immunoprecipitation and treatment of cells with neuraminidase (manuscript #4). We also observed differences in reactivity of sera for glycosylated and non-glycosylated HIV-1 gp120 glyco protein that displays LeY and related antigens (17). No reactivity of the anti-peptide serum was observed against fibrinogen, fibronectin, vitronectin and von Willebrand factor by ELISA. This was of concern since the RYD motif is similar to the RGD motif found on these molecules. This result is in keeping with other observations that the RGD motifs on the aforementioned molecules display different conformations and do not necessarily induce anti-RGD antibodies that cross-react with all RGD motifs (41). We showed previously that a RYD peptide specifically interacts with the platelet protein GPIIb-IIIa and not with tumor cells that express other integrins (42, 43). Nevertheless, it is possible that our sera is reacting with the human breast carcinoma antigen BA46; immunoprecipitation profiles indicate a protein at 46KDa (Manuscript #4). BA46 contains an RGD tract in its EGF-like domain and antibodies to this site have been used to mediate tumor regression in mice (44). However, we observed that anti-sera directed toward the motif YYRGD, does not immunoprecipitate glycoproteins in the cell lysates as sera elicited by immunization with YYRYD or YYPYD motifs (manuscript #4).

Functionality of anti-peptide sera for tumor cells

A basic hypothesis of our studies is that peptide mimotopes can induce humoral responses that are functional and specific for tumor cells. The following points favor opinions that humoral responses mediate critical effector mechanisms that contribute to tumor immunity. 1.) Antibodies to tumor associated antigens in general appear to have a favorable prognosis that is statistically significant; 2.) CDC and ADCC effector mechanisms are attributed to the efficacy of MAb adoptive therapy in patients; and 3.) The administering of IL-2 to patients is considered to activate effector cells for ADCC. Consequently, we view these effector mechanisms as a biologically important in vitro assessment of the functionality of immunological mimicry. CDC has more activity against melanoma cells than cytotoxicity associated with various effector cells (45). Moreover, melanoma cells can express sLeX. Anti-P3 sera displays some binding to sLeX synthetic probes. We speculated that the melanoma lines might express sLeX as previously suggested or the sera cross-reacts with the sialyl moiety on GD2/GD3. We performed FACS analysis with the anti-sLeX antibody FH-6 and observed binding of this MAb to these melanoma cell lines (data not shown). Subsequently, we have examined CDC mediation of various sera raised to peptides against melanoma cells as controls to assess functional specificity, and against human tumor cell lines that express LeY. In the past we used rabbit complement (16). In more recent studies we have used human complement (human serum diluted 1:3).

In initial assays, murine sera generated against selected peptide-proteosome conjugates (Table 2) were tested for tumor cell reactivity and CDC as previously described (28). In Table 5, peptides P1, P2 and P3 all showed an ability to mediate CDC of the LeY expressing human breast lines SKBR3 and MCF-7 and the human ovarian line OVAR-3 similar to the positive control BR55-2 MAb. BR55-2 does not mediate CDC of the human melanoma lines SKMEL-28 or WM793 line. P2 and P1 mediated CDC of the human melanoma lines close to non-specific values using control sera (C1), but P3 displayed moderate CDC activity. These data suggest that despite the broad specificity of the sera for carbohydrate constituents by ELISA, the respective sera recognize ubiquitous carbohydrate subunits differently when expressed on cells. These data subsequently indicate that sera generated to carbohydrate mimicking peptides can recognize important tumor associated antigens with a high degree of specificity.

Table 5. Summary of Complement Dependent Cytotoxicity Results

Tumor	C1	P2	P1	P3	G1	G2	B1	LeY-PAA	ME361	BR55-2
SKMEl-28	3	13	10	32	75	87	10	4	53 (50µg)	3 (100µg)
SKBR3	6	80	90	86	10	13	90	20	10 (100µg)	80 (100µg)
MCF-7	3	29	66	56	20	15	70	26	5 (50µg)	75 (100µg)
WM793	5	9	9	28	90	90	10	2	63 (30µg)	1 (100µg)
OVAR-3	5	84	89	86	9	11	85	25	6(50µg)	80 (100µg)

Values are averaged percent cytotoxicity. Final dilutions are 1:15 for sera. Monoclonal antibody ME361 and BR55-2 concentrations are per ml.

As a further control, we immunized Balb/c mice with LeY-PAA, which is a multivalent LeY form on Polyacrylamide (PAA) (available from Glyco Tech Inc.), adsorbed onto bacteria (Salmonella minnesota) and incorporated into proteosmes. Consistent with published results (46) immunization resulted in an anti-LeY immune response measured by ELISA. In addition, we observed that the multivalent LeY-PAA form induces anti-sera reactive with tumor cells, displaying CDC mediation at low levels. These data indicate two things. 1) that multivalency is of importance in generating responses to natively expressed LeY on tumor cells and 2) that the peptides do a better job of inducing sera that mediates CDC. Our finding that LeY-PAA induces an immune response that reacts with native LeY expressed on tumor cells provides us with an opportunity to compare functional immune responses between the carbohydrate and peptide antigens.

Phage display peptides and anti-tumor reactivity.

A basic hypothesis of this application is that peptide mimotopes identified from screening peptide phage display libraries with anti-carbohydrate antibodies can prove specific in eliciting a humoral response directed toward the respective tumor. We have identified a number of peptides from a 15 mer phage library reactive with the anti-LeY antibody BR55-2 (manuscript in preparation) and the anti-GD2/GD3 specific antibody ME361. The B1 (Table 2) peptide is representative of a peptide isolated by BR55-2 and G1 and G2 (Table 2) are representative of peptides isolated by ME361. We chose these two potential GD2/GD3 mimicking peptides because of similarities with peptides identified that mimic the histo-blood group antigens (data not shown). Subsequently, comparisons between G1 and G2 peptide sequences that mimic GD2/GD3, with peptides that mimic the histo-blood group related antigens, can provide information about how amino acid differences lead to specific antigenic and immunological mimicry.

We immunized Balb/c mice with these peptides in the proteosome form and examined induced sera in a variety of assays relevant to this application. Sera to B1 cross-reacts with synthetic LeY and sera binding to B1 peptide coated plates was inhibitable by LeY (manuscript in preparation). Sera elicited by B1 binds to LeY expressing cells and mediates CDC to LeY expressing human tumor lines but not to ganglioside expressing human tumor lines (Table 4). In related experiments, sera to G1 and G2 peptides is GD3 inhibitable (data not shown) and binds to two melanoma cell lines as assessed by FACS and CDC (Table 4). CDC killing was specific to melanoma lines and superior to that observed for the P1, P2, P3 anti-sera. As controls, the monoclonal ME361 mediated WM793 and SKMEL-28 cell killing up to 100% at 30ug/ml Ab concentration (data not shown), a finding consistent with previous results. As a negative control, the monoclonal BR55-2 displayed marginal CDC activity for the melanoma lines. While only two melanoma lines are shown in table 4, the lack of a functional humoral response to 3 LeY expressing cell lines indicate that the anti-G1 and G2 functional response is specific for gangliosides.

These results suggest that 1.) Peptides can be identified by screening phage libraries that can be immunogenic to mediate a functional humoral response in vitro. 2.) That induced CDC can be specific for particular cell lines. and 3.) That peptide mimics can be effective as effective or more effective than the carbohydrate that they mimic in inducing CDC immunity. These initial data set the ground work for improving upon peptide immunogenicity and specificity, as well as examining the underlying immunological mechanisms for functional mimicry.

Molecular basis for peptide binding to anti-LeY antibodies

The B3 antibody binds to the peptide sequence APWLYGPA presented on phage display in which the putative pentapeptide sequence APWLY is critical for binding to the antibody (47). To establish how this putative sequence mimics LeY binding to B3, we "fit" the pentapeptide sequence into the B3 combining site (manuscript #1 in appendix) using the program LIGAND-DESIGN (LUDI (48) Biosym Technologies). This program searches a molecular library for fragment's representative of the amino acids in the target peptide sequence. The program then positions the fragments within the combining site devoid of steric conflicts. The APWLY sequence was modeled such that the Trp, Tyr, Leu and Ala residues occupied relative positions as the identified LUDI fragments. The judicious positioning relied upon intermolecular interaction calculations in which several potential binding modes of the peptide were ranked according to the stability of the complex. In the most stable conformation, we observed that the AP residues occupied a similar position to the LeY GlcNAc residue. This positioning indicates that the proline residue mimics the spatial position of the glucose unit of GlcNAc, while the Ala methyl group is positioned similarly as the terminal methyl group of GlcNAc's N-acetyl. The Trp residue occupies a volume associated with the Fuc 1-3 moiety , and the Leu residue occupying

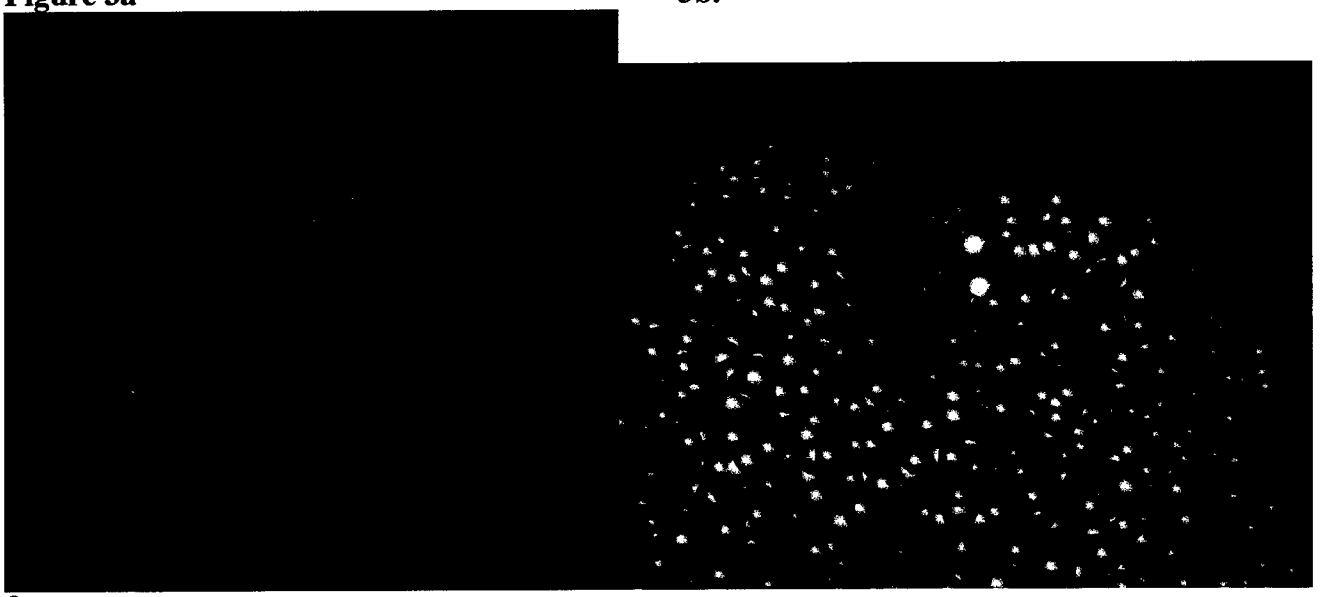
the volume and hydrophobic interaction of bGal. The Tyr residue occupies a position not associated with LeY binding to B3. We observe that the low energy binding mode conformation adopts a turn region similar to that observed for the YPY motif in binding to ConA (49). This conformation lends itself to the Tyr residue of the peptide to potentially interact with several residues in CDR2 of the heavy chain of B3 that include Asp H53, Ser H52, Ser H55, or Ser H56. These residues are different in BR55-2, which does not bind the monovalent APWLYGPA peptide in a series of ELISA assays (manuscript in preparation). Energy optimization of the positioned peptide identified similar functional groups within the B3 combining site in contact with the peptide and carbohydrate tetrasaccharide core of LeY. This analysis therefore defines a strategy for determining the molecular basis for antigenic mimicry of particular motifs, providing a unique perspective of how a peptide sequence fits into the antibody combining site, competing with a native antigen.

We extended our approach described above to determine what kind of motifs could bind to BR55-2 and whether we could isolate such motifs with BR55-2 from a peptide phage screen (manuscript in preparation). If we could identify motifs this way then we may be able to improve upon antigenic mimicry for LeY. We know YPY, YRY and WRY motifs are mimics of various carbohydrate subunits. Searching the LUDI database using the modeled structure of BR55-2, we identified these motifs as interacting with BR55-2 (data not shown). In the LUDI search, we also identified a non planar-X-planar type motif, FSLLW, as a possibility (Figure 3). In figure 3a non-overlapping residue types were identified using the LUDI program as in our B3 studies. In figure 3b, a space filling rendering of BR55-2 with optimized FSLLW peptide in the BR55-2 combining site is shown.; and in figure 3c an optimized FSLLW peptide in the BR55-2 combining site is shown in contrast to LeY positioned in BR55-2 combining site. The topological similarity is very good.

We next screened a 15mer peptide library with BR55-2. Analysis of peptide sequences identified by panning the 15 mer library (Figure 4) with BR55-2 indicates that the central Planar residue-X-Planar residue type tract is represented in families 12, 14-17, 20 and 21. In family 21, an inverted tract WPYL is observed in comparison to the sequence PWLY found by B3. A sequence tract of WRY is observed in Family 17. We also observed an FSLLW tract in family 18 for BR55-2. This result indicates that we can define motifs de novo for prediction that are equivalent to those identified experimentally from a phage library.

We showed that sera elicited by the proteosome form of this peptide (B1 Table 2) specifically mediated CDC (Table 4). To further evaluate the specificity of the B1 peptide we synthesized B1 as a MAP form and immunized Balb/c mice, with QS-21 as adjuvant and examined its reactivity patterne with various Le syntheic probes by ELISA (Figure 5). The utility of MAP peptides is in their apparent advantage as immunogens (50, 51). For example, MAPs have proved to retain all the immunological properties of an intact anti-id upon which the peptide was based (50), and was found to be qualitatively similar and quantitatively superior to the linear monomeric 15mer anti-Id derived peptide (50). However, the MAPS described above (50,51) contain T cell epitopes within the peptide sequence. MAPS that do not contain T cell epitopes do not induce significant IgG titers (52). We have observed this trend with MAP peptides (manuscript #4).

Figure 3a **3b.**



3c.

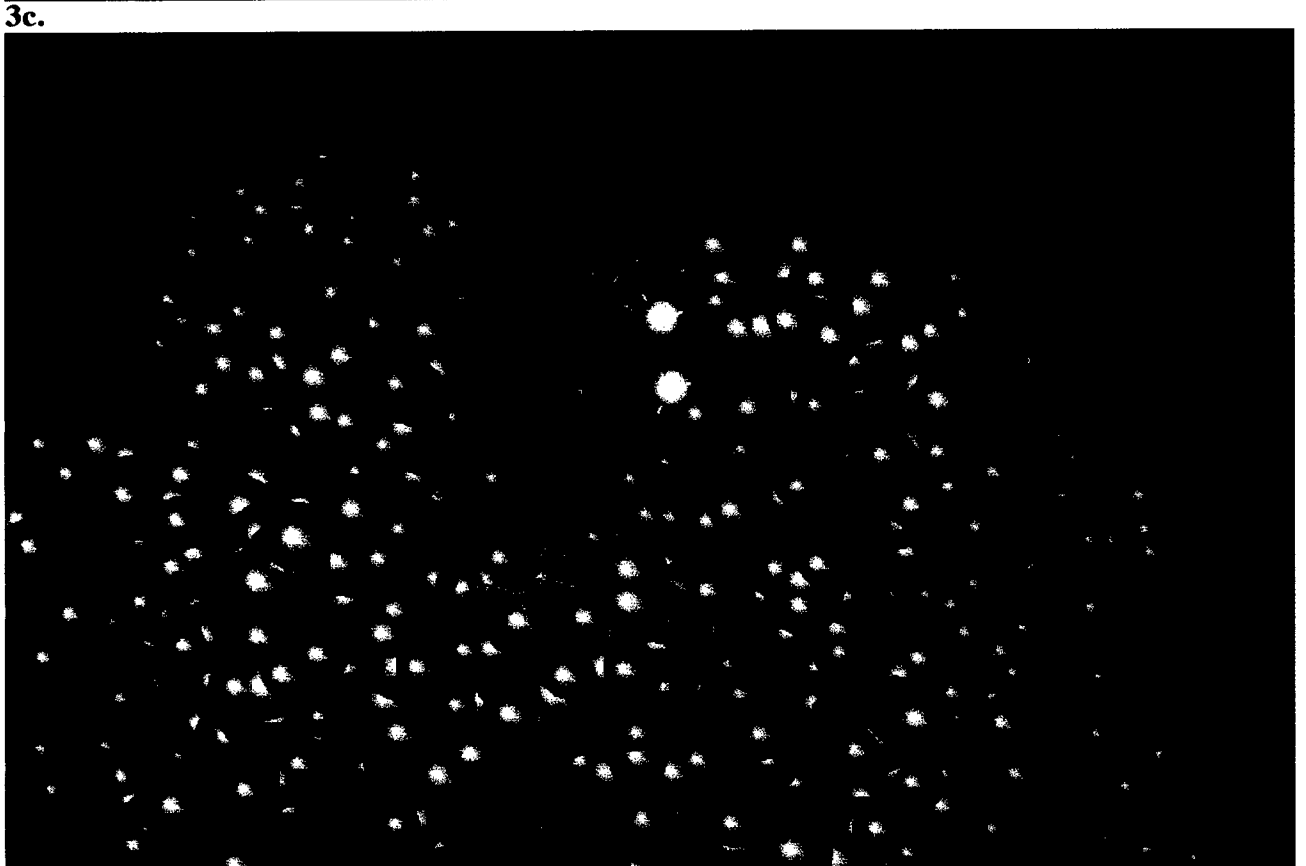


Figure 3. Identification and placement of the FSLLW motif interacting with BR55-2. **3a.** non-overlapping residue types where identified using the LUDI program as in our B3 studies. Phe is orange, Leu type residues are colored yellow and cyan, Trp is colored green, BR55-2 is colored purple. Ser residue functional group is associated with the yellow colored lipophilic Leu type group. **3b.** space filling of BR55-2 (gold color) with optimized FSLLW peptide in the BR55-2 combining site. Methyl groups on Leu residues colored yellow and green. **3c .** optimized FSLLW peptide in BR55-2 combining site in contrast with LeY (colored blue) position in BR55-2 combining site.

Figure 4. Multiple alignment of isolated peptide families reactive with BR55-2

Family	Phage ID	Sequence (1 … 36)
2	BR55-posSEQ09	....T WPVVHGACRA HGHC..
6	BR55-posSEQ15	..... .GLDLLGDVR IPVVRR.... .....
7	BR55-posSEQ02	..... .GLDLLGDVR IPVVAS.... .....
8	BR55-posSEQ28	....SLVSSLD IRVFHRLP.. .....
9	BR55-posSEQ31	.....V GITGFVDPLP LRLL..... .....
10	BR55-posSEQ29	..... GAFSSPRSLT VPLRR..... .....
12	BR55-posSEQ17	....AGR**WV** **F**SAPGVRSIL .....
14	BR55-posSEQ12	.....H GRFILPWW**YA** **F**SPS..... .....
15	BR55-posSEQ30	.....F AR**YLF**THWWR LPVD..... .....
16	BR55-posSEQ21	..... .R**YLF**YSVHP WRVSYS.... .....
17	BR55-posSEQ26	..... ARVSF**WRY**SS FAPTY..... .....
18	BR55-posSEQ34	.....I MILLIFSLLW FGGA..... .....
20	BR55-posSEQ13	.....GRV ASMFGGYFFF SR..... .....
21	BR55-posSEQ14	**WPY**LRFSPWV VSPLG..... ....
22	BR55-posSEQ10	.....TSV NRGFLLQRVS HP..... .....
23	BR55-posSEQ32	....ARFR HSTKSAQFVP L.....

The anti-B1 sera was predominately of IgM isotype as observed in our previous studies. The sera displa a three to fold increase in reactivity for LeY over Leb, tittering up to 1:2000 in ELISA (data not shown). At 1 serum dilution (Figure 4), we observed about the same level of reduced reactivity for Leb hexasaccharide, L pentasaccharide, sLeX, Lea, and sLea, while higher levels of reactivity are observed for LeY and LeY constitue Minimal binding is observed for a ubiquitous disaccharide unit Galb1-3Gal. This lack of reactivity is in contras that observed with sera generated to our general aromatic-aromatic motifs (manuscripts #2,4). The best reactivit observed with the LeY constituent Fuca1-3GlcNAc (Figure 1). The anti-B1 sera appears to recognize both l moieties on LeY since LeX displays diminished reactivity. The anti-B1 sera distinguishes the Fuca 1-3 and GlcNAc linkage, displaying significantly reduced reactivity with Fuca1-4GlcNAc. This selective interaction apart reactivities between Leb and LeY, since reactivity is observed for the H type 1 constituent of Leb. Th results suggest that we can develop peptides theoretically and by phage isolation that rendered as clustered M peptides, are powerful immunogens displaying the desired specificity for tumor antigen by interacting with a spec epitope.

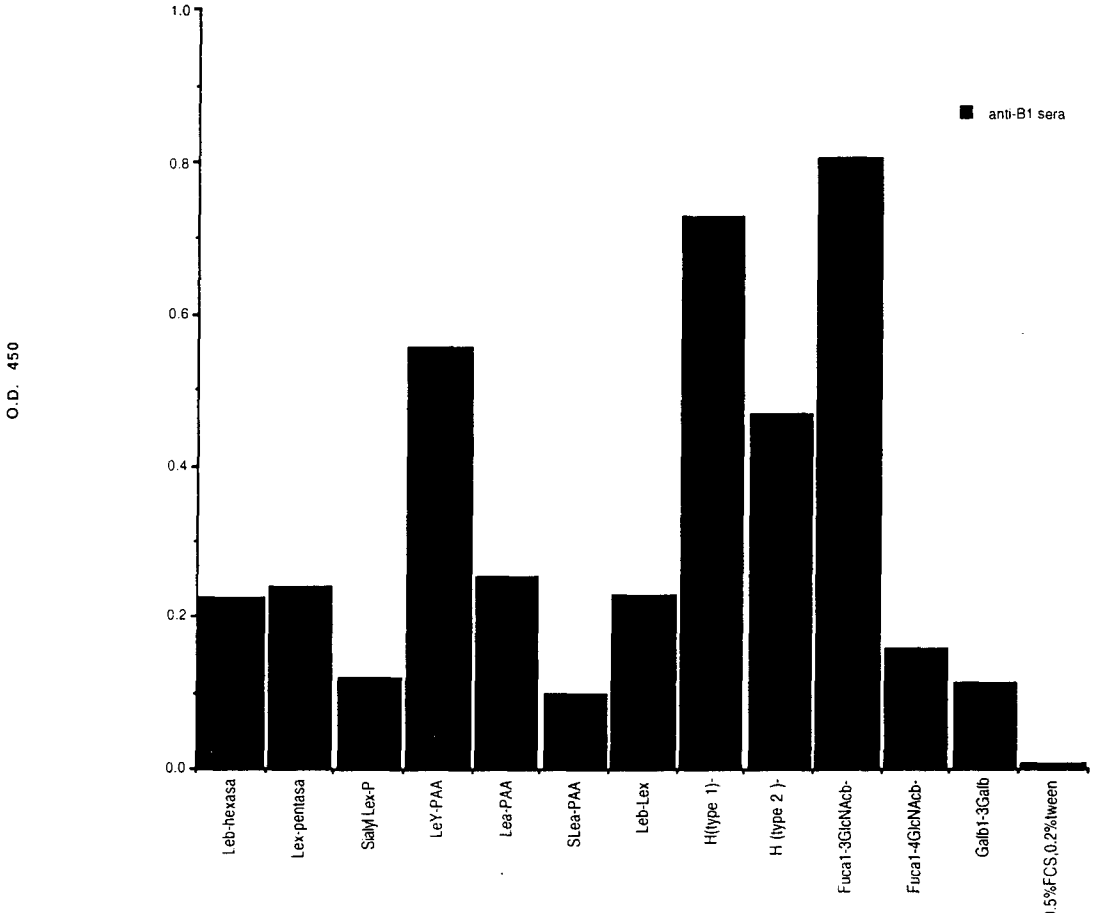


Figure 5. Specificity in binding among Le antigen probes and constituents by anti-B1 MAP derived sera.

Progression of studies during the coming year.

Kinetic studies. The critical feaatures for the interaction of carbohydrates on the cell surface are multivalencey and mobility. Valence plays the key role in protein-carbohydrate interactions, therefore it is important to use methods that allow for the monitoring of these effects. For example the increased valency of the antibody from one to two results in 100-fold slower dissociation of the antibody from the carbohydrate antigen. Analysis by surface plasmon resonance of the influence of valence on the ligand binding affinity and kinetics of an anti-carbohydrate antibodies have been described. We are using a surface plasmon resonance (SPR) method for the study of BR55-2 with the generated peptides. The methods involve antibody or LeY-PAA captured on BIACORE sensor chip followed by peptide binding or inhibition.

For inhibition studies LeY-PAA is captured on the chip and various concentrations of peptides are mixed with MAb or polyclonal sera prior to injection over captured LeY surfaces. In contrast to conventional assays, the method provides kinetic and affinity data and real time monitoring without any requirements of labeling of the reagents or the use of secondary detection reagents. SBR technique allows to study the kinetic parameters such as association and dissociation rate constants. Kinetic data can provide the necessary characteristic of the mimetics in terms of affecting high affinity interactions. The multivalency results in higher affinity binding for carbohydrate binding protein but also in more complex interaction than is one to one model. The studies of detail kinetic parametes will allow comparison of the kinetic parameters such as on and off rates and Kd values for monovalent and MAP peptides.

To increase the affinity of BR55-2 for LeY by site-directed mutagenesis: Site-directed mutagenesis permits modification of the functional characteristics of specific proteins. since the amplifying primers used in the polymerase chain reaction (PCR) are incorporated into the product, PCR can be used to modify the ends of DNA segments.

Approach: Both double-strand insertional mutagenesis using Recombinant Cycle Polymerase Chain Reaction (RC-PCR) and single-strand site-directed mutagenesis will be applied. **Insertional mutagenesis:** we will use RC-PCR to fulfil this task: amplify and mutate 3 ng of plasmid as template in two seperate PCR amplifications, by adding 25 pmols of each primer, 1.25 units of ΔTaq enzyme, 400 μM of each dNTP, 100 mM KCl, 20 mM Tris-HCl, pH8.3, 2.5 mM $MgCl_2$, 1.5 μg galatin. Total volumn of the reaction is 50 μl. Primers have 5' ends that are composed of the complementary strands of the segments that is to be inserted. The PCR cycle profile is as follows: 94°C, 30"/ 52°C, 30"/ 72°C, one minute per kb, total 18 cycles, followed by 72°C, 7' as final extension. After PCR, seperate each entire PCR product from supercoiled plasmid template by running a 1 % agarose gel followed by glass bead extraction. Following the combining, denaturing and reannealing of the two PCR products, double-stranded products will form with single-stranded ends that are complementary to each, and will anneal to yield recombinant circles. These constructs are transformed into E.Coli strain HB2151, a strain with amber stop codon suppresion defficient, through electroporation to increase its typical low rate of transformation in this type of mutagenesis. The transformants are plated onto ampiciline containing LB plate and culture at 37°C overnight. Because there is aways the possibility of a sequence error in a single clone following PCR amplification even with high fidelity Taq enzyme, we will either choose to test more than one clone in a functional assay or clone a resctrition fragment containing the mutated or recombined region of interest into a construct that has not undergone PCR amplification. Since all our antibody fragments of interest are cloned into the phagemid, we can use an improved oligo-directed mutagenesis method on single-strand vector DNA to generate mutation.

To develop multivalent and multispecific antibody agents for increased tumor reactivity.

Rationale: In view of the high penetration of capacity of scFV into tumors, they should be particularly useful for antibody-directed chemotherapy of cancer. An essential disadvantage however of scFVs is the monovalency of the product that precludes polyvalent binding to its antigen providing an avidity factor to increase its affinity. Dimerization motifs described earlier make the final scFv constructs larger, potentially immunogenic and therefor unsuitable for the production of bivalent antibodies. There is a need therefore to develop other methodologies for the production of bivalent antibodies in E. coli. It would also be advantageous if an scFV could be chemically coupled to various reagents via their C-terminus, as is possible with Fab' fragments, which have free C-terminal cysteines. This would also facilitate the creation of bivalent homo-and heterodimers by site-specific dimerization in vitro as shown for recombinant Fab' and FV'. Bivalent antibodies bind multimeric antigens with higher avidity and bispecific antibodies can cross-link two antigens-for example, in recruiting cytotoxic T cells to mediate tumor killing.

Construction of dimeric and/or trimeric scFV molecules: Overall, the structure of the dimeric recombinant DNA molecule will contain the following components in order from N-terminus to C-terminus: the 5'-Sfi I-scFV fragment, linker, scFV-streptavidin (SA)-Cystein-$(His)_6$-stop codon-Not I-3'. the 5'-Sfi I -scFV fragment and the scFV-streptavidin-Cystein-$(His)_6$-stop codon-Not I-3' fragment are generated seperately. The whole linker sequence is devided into two and incorporated into the relative primer for annealing the two fragments. 5'-Sfi I-scFV fragment is amplified individually through PCR using our clone Amhk 13 as template. the 3'-primer designed for this amplification contains a portion of the linker sequence at 5' end which is going to be annealed to the rest of the linker sequence which was built into the 5' primer for the amplification of scFV-streptavidin-Cystein-$(His)_6$-stop codon-Not I-3' fragment. To generate the scFV-streptavidin-Cystein-$(His)_6$-stop codon-Not I-3' fragment, we amplify the scFV first with a 3' primer which contains the 5' end sequence of SA. We then amplify the streptavidin-Cystein-$(His)_6$-stop codon-Not I-3' fragment with a 5' primer containing the 3' sequence of the scFV fragment and a 3' primer containing the entire tail of Cystein-$(His)_6$-stop codon-Not I-3'. The two fragments are then annealed to each other to form the second fragment, i.e. the scFV-streptavidin (SA)-Cystein-$(His)_6$-stop codon-Not I-3' fragment. The final construct contains Sfi I and Not I restriction sites at its 5' end and 3' end which facilites itself to be ligated to the expression vector pCANTAB 5E. To generate the trimeric molecule from the dimeric molecule, another PCR amplification of the scFV fragment needs to be

performed with Sfi I sites built into both the 5' primer and the 3' primer. This product is annealed to the N-terminus of the dimeric scFV molecule then ligated to the same expression vector. Under this construction, the Pel B sequence is no longer applied.

Expression of the dimeric and/or trimeric scFV molecules: pCANTAB 5E expression vector contains a gene III signal sequence. So our expressed product could be either secreted into the culture media or into the pariplasmic layer or stays in the cell cytosole. To determine the expression pattern, cell culture supernatent, periplasmic extract and whole cell extract are need to be tested. In brief, to prepare the peri-plasmic extract, resuspend thecell pellet in 1x TES buffer, then add an equal volumn of ice-cold 1/5 x TES and vortex. Incubate on ice for 30'. Spin the whole contents in a 1.5 ml tube at 14,000 rpm at 4°C for 10'. Collect the supernatant which contains the scFV fragment from the pariplasmic layer. The whole cell extract is made by ultrasonication. When a positive clone has been selected, do a time course for determining the optimal expression. The time course is going to be carried out at 28°C at which it is believed that the formation of the inclusion body is minimized. To maximize the expression, we use baffled flasks. The feature of fast growth and expression has made the bacteria expression system a good candidate for evaluating the best insert structure when multi-clones are compared. When functional assays reveal the best clone, we will further subclone the fragment into Pichia yeast expression vector pPIC9 (Invitrogen). The resulting construct is then linearized by restriction digestion with Bgl II to allow efficient integration into the Pichia cell genome. Transformation is carried out through electroporation. A recombination event occurs in vivo between the 5' and 3' AOX1 sequences in the yeast vector and those in the Pichia genome. The result is replacement of AOX1 with the gene of interest. All the transformants are transferred to histidine-deficient (His) solid agar plate. Cells in which recombination has occured will grow. Others will not produce histidine and will grow very slowly. Screen for integration at the correct locus. Select those which exhibit slow growth on MeOH no longer contain the AOX1 gene and have a $His^+ Mut^s$ (MeOH utilization-slow) phenotype. Pick 20 $His^+ Mut^s$ clonies to grow in medium containing glycerol first followed by growing cells in Methanol-containing medium but without glycerol for 5 days at 28°C in baffed flask. Culture supernatant is collected to undergo purification. At this point, the $(His)_6$ tag will be removed first from the dimeric or trimeric fragments. To increase the expression yield, a multi-copy featured expression will also be performed using a unique expression vector pA0815 which is specially designed to generate multiple copies of the gene of interest in a single vector. With our previous experience, this system probably is the best one so far in terms of its yield, biosafety, and stability.

Characterize the in vitro and in vivo cellular responses to tumor cells induced by carbohydrate mimicking peptides.

Rationale: It is hypothesized that peptides can mediate anti-tumor cellular responses. In this aim we want to establish the role of T cells in conveying anti-tumor effect in our Meth-A tumor studies. In our preliminary studies we observed that the P4 mimotope for sLeX (Table 2) induced a significant anti-tumor effect against sLeX expressing Meth-A cells in syngeneic Balb/c mice (Table 3). Elicited antibody is detected against P4, but cellular responses have not been investigated. We would like to understand the nature of the apparent in vivo anti-tumor response; the response could consist of only humoral or both humoral and cellular components. In our tumor challenge experiments, P4 conjugated to proteosomes was used as immunogen. This formulation generates predominately IgG. Therefore, we know that T cell response is mediated by this formulation. The MAP form of P4 elicits predominately an IgM response. Exogenous antigens are thought to be processed and presented predominately on association with MHC class II molecules for stimulation of CD4+ T cells. The interaction of the TCR with the immunogenic peptide bound to MHC class II molecule leads to the activation of the CD4(+) T helper cells. T helper cells are divided into two subsets, Th1 and Th2, on the basis of cytokine secretion. Th1 cells that secrete IL-2, IFN-gamma and tumor necrosis factor-beta induce DTH, while Th2 cells secreting IL-4, IL-5, IL-6 and IL-10 induce humoral immune response. However, the mechanism of selective activation of Th1 and Th2 cells in response to different antigens is not fully understood. Consequently, we want to generate T cell lines and clones that are reactive with the P4 peptide and determine if they are reactive with synthetic sLeX and sLeX expressing tumor cells.

If we are successful in isolating T cell lines or clones that are specific to carbohydrate, we can use these lines or cells for in vivo analysis of their activity, by transferring these cells to naive mice and challenge with Meth-A tumor. The reconstitution of tumor immunity in niave mice by these T cell lines or clones would define the role played by T cell mediated immunity. In addition, we can passively transfer anti-P4 serum generated in

aim 2 to naive mice alone, or along with the T cell lines found in this aim, to further assess the functional role of these two arms in anti-tumor immunity.

Approach: We want to establish the characteristics of T cell lines and clones isolated from lymph nodes and spleens of mice immunized with the P4-proteosome formulation that render an anti-tumor effect and compare these responses with T cells derived from mice immunized with P4-MAP. We will 1) Perform bulk assays for T cell proliferation against sLeX expressing tumor cells, control cell lines, synthetic sLeX-PAA, P4 peptide, and irrelevant control peptides. 2.) Perform Bulk analysis for anti-peptide and anti-tumor CTL. 3.) Perform DTH against sLeX Meth-A tumor cell. 4.) We will also develop T cell lines and/or clones to more precisely define interactions with the P4 peptide compared to interactions with sLeX expressing target cells. These lines or clones will be used for in vivo challenge model. Collectively, these assays will tell us about the general characteristics of Th1 and Th2 responses induced by the P4 peptide.

In our first set of experiments, the antigen-specific proliferative capacity of T cells from immunized and control mice will be analyzed. Cultured cells will be activated either with sLeX expressing irradiated tumor cells, synthetic soluble sLeX-PAA, P4 peptide (linear and MAP form), or with irrelevant control peptides. Incorporation of 3H-thymidine into cells will be measured. In addition, supernatants will be tested for the presence of Th1 and Th2 lymphokines. Long term T cell lines will also be generated. Clones will be prepared by limiting dilution and expanded in vitro. These clones will be tested by FACS analysis for the expression of CD4 and CD28 or CD8 and CD28. Again supernatants will be tested for lymphokine profiles.

The biological activity of these clones or lines will be tested by injecting them into naive animals, followed by challenge with the Meth-A tumor. In addition, we will also analyze Th2 helper activity, by examining B cell proliferation. Most likely anti-tumor responses are being mediated by DTH or ADCC. However, it is possible that CD4+ CTL, NK, or CD8+ CTL could be involved in this process. Therefore, we will try to generate bulk CTL against tumor expressing sLeX. In this assay we will use as stimulators either P815 tumor cells pulsed with P4 or sLex tumor cells. Both cultures will be tested against peptides or carbohydrates. As peptide targets we will use P815 pulsed with p4 and as carbohydrate target we will use sLeX expressing Meth-A. As a control target we will use P815 alone. If we see any CTL activity using anti-CD4 or anti-CD8 monoclonal antibodies we will eliminate appropriate population of T cells and we will analyze CTL activity.

In the DTH assays, a classical skin test will be performed. Balb/c mice immunized with P4-proteosome or irrelevant control peptide formulation will be challenged with irradiated Meth-A tumor cells or irradiated control tumor cells in the ear. Ear thickness difference is the readout for this assay.

Challenge and Priming, Boosting Studies:
As in our pervious Meth-A challenge experiments which is a prophylatic vacciantion approach, groups of Balb/c mice are immunized i.p. with P4 -MAP or proteosome-P4. Ten days after the third immunization, mice are challenged s.c. in the foot pad with 10^6 live Meth-A cells expressing sLeX antigen (day 0). Sera will be collected prior to challenge and every 2 weeks there after and assayed for isotype distribution, CDC and ADCC activity. In related experiments we will firsts immunize with P4-proteosome for the first immunization, followed by subsequent boosts (2) with P4-MAP peptide. These groups will then be challenged with Meth-A tumors. The opposite immunization will also be performed; immunizing first with P4-MAP, followed by 2 boosts with P4-proteosome followed by Meth-A tumor challenge.

As a therpaeutic vaccination approach, we will immunize groups of Balb/c mice sc with 10^6 live Meth-A cells expressing sLeX antigen (day 0) and begin follow up peptide immunizations at day 0, 1 week, 2 weeks and 3 weeks after the primary tumor administration. Groups will be hyperimmunized with 100ug of P4-proteosome ip weekly. Mice usually die 1 to 1.5 months after tumor administration without treatment (Table 3). For the group starting at day 0 we expect to immunize the group 4 times (over 4 weeks). In the second group, we expect to immunize over three weeks and so on.

Tumor growth in animals. Thymus-deficient nude mice (nu/nu, Balb/C background) 6 to 8 weeks old will be inoculated subcutaneous (sc) in the flank or under the skin of the neck with $1x10^5$ human tumor cells (SKBR3, MCF7 or WM793, SLMEL-28 as controls). Serum from immunized mice will be injected ip at various dilutions and times to test their ability to inhibit tumor growth. Alternatively, the tumor cells ($5x10^4$) will be admixed with sera and immediately 0.2 ml of these suspensions will be injected ip. Experimental groups will

consist of 4-5 mice. The statistical significance of differences between the groups is determined by applying Student's two tailed T test.

Conclusions

The interplay between carbohydrates and proteins is of fundamental importance in a number of different biological processes. In particular, cell adhesion and cell recognition events are often mediated by protein-carbohydrate interactions forming a basis in the etiology of tumors. Aberrant glycosylation may be crucial in tumor progression, since cells acquire competence for metastasis and a faster clonal growth via newly synthesized carbohydrate structures. The carbohydrate expression patterns differ according to clinical features and are also changeable in clinical courses of respective tumors types. The differential exposure at the cell surface of specific carbohydrates may have implications for cell-protein or cell-cell interactions and for antibody-directed tumor detection and therapy. To date, the functional role of the Lewis antigens have not been fully explored. Presumably, these carbohydrates represent functional structures or determinants of molecules controlling cell motility, adhesion and proliferation, functions related to the metastatic potential of human cancers. Subsequently, antibodies that are directed to such antigens might prove useful as therapeutic agents either directly or as carriers for other agents. We have shown that LeY is recognized in a similar fashion by sevarl anti-LeY antibodies. This information can lend to developing high affinity anti-LeY antibodies that might prove useful as reagents to target LeY in passive therapy approaches.

Antibodies are also known to function as surrogates or mimics of ligand binding sites on receptors, binding to both receptor agonists and antagonists. We have shown that peptides can function as adeauate mimics of LeY and realted Le antigens also expressed on tumor cells. We have shown that these peptides can be immunogenic and we are know poised to optimize such peptides that might prove useful in a vaccine strategy for breast cancer.

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Molecular Recognition of a Peptide Mimic of the Lewis Y Antigen by an Anti-Lewis Y Antibody

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Abstract

Peptides as mimics of carbohydrates display a distinct advantage in vaccine design because of ease of synthesis and their inherent T cell-dependent nature as immunogens. While peptides that mimic carbohydrates have been described, it is not clear how they do so. To further our insight into structural relationships between peptide-mimics and carbohydrate structures, we have analyzed a potential recognition scheme between the murine monoclonal antibody, B3, directed against the tumor-associated antigen Lewis Y oligosaccharide and a peptide identified from phage display screening with B3. The Lewis Y core antigen is a difucosylated structure consisting of four hexose units. The B3 antibody binds to the peptide sequence APWLYGPA in which the putative sequence APWLY is critical for binding to the antibody. Not having experimental structural information for B3, the crystal structure of another anti-Lewis Y antibody, BR96, solved in complex with a nonoate methyl ester Lewis Y tetrasaccharide, provides a molecular basis for LeY antigen recognition and specificity, and how this binding relates to peptide binding. As a guide to place the APWLY motif in the B3 combining site, a fragment library was searched for analogous compounds that have the potential to bind to B3. Our modeling study shows that the B3-peptide complex shares similar recognition features for the difucosylated type 2 lactoseries' structure. This analysis provides a molecular perspective for peptide mimicry of a carbohydrate epitope.

Introduction

The interplay between carbohydrates and proteins is of fundamental importance in a number of different biological processes. The roles of carbohydrate structures present on the cell surface range from influencing tumor growth, progression and metastases, to mediating bacterial and viral attachment (1-3). Subsequently, the inhibition of these interactions is a possible point of therapeutic intervention for a number of diseases. The definition of consensus carbohydrate structures on glycolipds or glycoproteins can serve as potential therapeutic targets (4). Carbohydrates are, however, difficult to synthesize (5, 6) and are generally poorly immunogenic. Defining carbohydrate mimetics, or surrogate antigens, might provide an alternative approach to overcome such drawbacks.

One type of mimetic is peptides. Evidence for the ability of a peptide or polypeptide to mimic a carbohydrate determinant comes from several sources (7-10). Anti-idiotypic antibodies have been defined that are mimics of carbohydrates, inducing immune responses that are cross-reactive with carbohydrate structures (8, 11-15). An appealing application for antibodies to identify carbohydrate surrogates, is the screening of peptide libraries with anti-carbohydrate antibodies. Screening against peptide display libraries identifies different molecular species than the one the antibody was raised against. Analysis of a larger repertoire of ligands reactive with an antibody combining site might establish structure/function relationships not evident from monoreactive molecules. Peptides that mimic carbohydrate structures have been defined in this manner (16-18). Peptides reflective of those found from screening libraries can induce immune responses cross-reactive with carbohydrate structures (19-22). These studies indicate that although antigenic mimicry of anti-idiotypic antibodies, or peptides, are accomplished using amino acids in place of sugars, the specificity pattern can be precisely reproduced.

The molecular basis for peptide mimicry of carbohydrate structures is not well characterized. For peptides as immunogens, it is important to distinguish the difference between chemical or antigenic mimicry, in which chemical similarity exists between the carbohydrate and peptide, and biological or immunological mimicry in which the mimetic induces particular carbohydrate reactive antibody subsets. In our efforts to elucidate how peptides might effectively mimic carbohydrate forms, we report here a

structural basis for antigenic mimicry of a peptide surrogate for the histo-blood group antigen Lewis Y (LeY). The LeY difucosylated type 2 lactoseries structure, Fucα1→2 Galβ1→4(Fucα1→3)GlcNAcβ →R, expressed on both glycoproteins and glycolipids, is one tumor-associated carbohydrate structure being explored as a target for monoclonal antibody (MAb) based imaging and therapy (23-27) and for vaccine development (25). Lewis Y is poorly immunogenic in humans. A peptide with the putative sequence APWLYGPA, as identified by phage display screening with the anti-LeY antibody B3, competes with the LeY antigen for B3 binding (18) and this and related peptides might prove to be effective in enhancing the therapeutic utility of a LeY-conjugate vaccine.

The B3 antibody displays homology with other anti-LeY antibodies (28), including the recently reported structure of antibody BR96, co-crystallized with a nonoate methyl ester LeY tetrasaccharide (29). The crystal structure of BR96 provides an avenue to elucidate possible recognition relationships among Lewis Y reactive antibodies. Molecular modeling of B3 complexed with the putative tetrasaccharide core of LeY was performed based upon the BR96-sugar recognition scheme (29). The B3 model emphasizes key polar and nonpolar interactions contributing to the molecular recognition feature for Le-Y shared among related anti-LeY antibodies, and consistent with mapping profiles of lactoseries derivatives reactive with B3 (30).

While current procedures for predicting ligand - antibody interactions are limited, mainly due to the conformational flexibility of ligands and antibodies and the role of solvent in mediating ligand recognition and binding, the utilization of a crystallographically determined starting position can, nevertheless, lend to discriminating differences in binding orientations of analogs. Using the positioned LeY structure in B3, we implemented the program Ligand-Design (LUDI (31) Biosym Technologies) to search a fragment library to guide in the position of the putative APWLY peptide sequence. Optimization of the positioned peptide indicates some preferences for B3 contact sites. This analysis therefore provides a unique perspective of how peptides, and perhaps anti-idiotypic antibodies, are cross-reactive with V domains specific for carbohydrate antigens.

Experimental

Model Building and Energy Refinement

The B3 structure was developed based upon the BR96 crystal structure 1CLY (29). The CDRs and the framework (FR) of the crystal structure template were mutated to those of the respective B3 heavy and light chains using Insight II. The side chain angles of the substituted residues were set according to angles identified in a database of side chains. Each CDR and framework region was changed individually, followed by 1000 cycles of energy minimization to eliminate close contacts between atoms. As in our previous studies, the program Discover (version 2.95 Biosym Technologies) was used for conformational calculations with the supplied consistent valence force field (CVFF) parameters. After model building, the respective structure was energy optimized to convergence. Molecular dynamics (MD) at 300 K and 600 K was used to further alleviate any close contacts during model building.

Initially a molecular dynamics simulation over 30 picoseconds using the program Discover was performed. The structure was then energy minimized using conjugate gradients to convergence. Following this initial equilibration, the calculation was resumed for another 30 picoseconds at 600 K at constant pressure and then cooled to 300 K over 50 picoseconds. During the second dynamics procedure, atoms lying further than 15 Å from all atoms of the CDR loops were held fixed. Non-hydrogen atoms of residues lying in the region 9 to 15 Å from all CDR loop atoms were harmonically restrained to their initial positions with a force constant of 30 kcal x mol^{-1} x rad^{-2}. These distance approximations result in fixing or restraining atoms of residues within the framework region of the antibodies. The backbone conformation torsion angles, phi and psi, of non-CDR loop residues were restrained to their initial values with a force constant of 1600 kcal x mol^{-1} x rad^{-2}. In addition, a torsional restraint of 10 kcal x mol^{-1} x rad^{-2} was employed around the omega bond. A time step of 1 fs was used. The resulting structure for B3 was again energy minimized using conjugate gradients to convergence.

Docking of Lewis Y to B3

The approach taken in the placement of the Lewis Y core in the antibody combining site made use of the position established from the BR96 crystal structure. After minimization, a molecular dynamics calculation over 100 picoseconds using the program Discover was performed. Restraints were imposed

to enhance the idealized hydrogen bonding pattern suggested from the heavy atom distances observed in the 2.6 Å map of BR96 (29). The dynamics run was not intended to be a detailed study, but to further alleviate any close contacts within the antibody and between the tetrasaccharide and the antibody. The calculation was initialized and equilibrated for 100 picoseconds at 300 K at constant pressure and resumed for another 50 picoseconds. The resulting structure was energy minimized using conjugate gradients to convergence. Charges and non-bonded parameters for the LeY structure were assigned from atom types from the CVFF parameter list supplied with Discover/InsightII.

Peptide placement within the B3 combining site

A LUDI search was performed using standard default values and fragment library supplied with the program to identify fragment positions within the B3 binding site. This program constructs possible new ligands for a given protein of known three-dimensional structure. Small fragments are identified in a database and are docked into the protein binding site in such a way that hydrogen bonds and ionic interactions can be formed with the protein and hydrophobic pockets are filled with lipophilic groups of the ligand. The positioning of the small fragments is based upon rules about energetically favorable non-bonded contact geometry's between functional groups of the protein and the ligand. The center of search was defined using the crystallographic LeY position. In this approach the OH-3^c position on the LeY structure was used as the sampling point. To identify possible fragments that are similar to the putative APWLY sequence, the radius of interaction, which defines the size of spheres in which LUDI is to fit appropriate fragments, was set as incrementing radii from 5 to 14 Å. Results of the search were compared with the putative peptide sequence side chain types, with those LUDI fragments retained within the B3-Lewis Y binding site which displayed similarities with the putative peptide side chains. The peptide was built using InsightII and positioned relative to the docked LUDI fragments. The peptide backbone and side chain torsional angles were rotated until the side chains of the peptide were approximate to the corresponding LUDI fragments. The peptide-B3 complex was subjected to energy optimization and molecular dynamics simulations as with the LeY-B3 complex.

Results

Structural properties of the BR96 template.

The crystal structure of BR96 provides a template to directly model the homologous B3 structure. B3, shares a high degree of sequence homology with BR96 and another anti-LeY antibody BR55-2. in both their light and heavy chains (Figure 1). The primary structure of the light chain of the anti - cholera toxin antibody TE33 (1TET) and the autoantibody BV04-01 (1CBV) display 92% and 87% identity respectively with the BR96 light chain (Figure 1). Both of these antibodies have been elucidated by X-ray crystallography as a complex with their respective ligands. Superposition of these two light chains with BR96 indicate that their CDR conformations are nearly the same except for CDR1 around the sequence tract "S-N-G" of BVO4-01 which is homologous with B3; RMS differences are 0.63 and 0.55 for BV04-01 and TE33 respectively excluding the CDR1 region. The conformational differences are either the result of sequence differences or due to induced conformations upon binding of the respective ligands. Crystal analysis of unligated BR96 suggests that CDR1L of BR96 undergoes a transition upon LeY binding (32).

For the heavy chain of B3, the IgG1 Fab' fragment B13i2 (2IGF) co-complexed with a myohemerythrin derived peptide, displays 77% identity with both BR96 and B3, having the same length CDR3. Superpositioning of these structures indicate that they display nearly the same conformations up to the CDR3 region (residues 1-92) with an RMS of 0.53 Å, suggesting CDR1 and CDR2 conformations have undergone similar conformational transitions if any. While the CDR3 length found in B13i2 is the same as that for BR96, not unexpectedly the conformations are different in the two crystal structures. The CDR3 loop in BR96 is typical of a type II turn with a Gly in the ith+2 position with the two Asp residues at positions 97 and 98 pointing outward from the antibody combining site towards solvent. Mutation of the native aspartic acid at position 97 in BR96 to alanine, results in increased tumor cell binding of BR96 (33). Interestingly, Ala is the native residue at this position in B3. Analysis of the nonoate methyl ester moiety of the complexed LeY tetrasaccharide suggest that this Ala could interact with a sugar residue extending from trifucosylated structures extended at the reducing site (our unpublished observation). This is consistent with the observation with another anti-LeY antibody AH-6 which binds equally well to LeY hexaosylsceramide, difucosylated LeY octaosylceramide and trifucosylated nonaosylceramide

suggesting that the epitope is limited to the LeY hexasaccharide or the extension at the reducing part of the oligosaccharide is incorporated into the binding groove without influencing antibody binding.

The BR96 based model was mutated to the B3 sequence and energy minimized. A relatively short dynamics run was performed to relieve any short contacts in the modeled structures. Molecular dymanics studies on antibodies indicate that major transitions in torsional angles are observed during the initial equilibration stage (34). It was not the intent to perform a detailed study of possible transitions of the CDR loops since in the "homology" modeled structure, hydrogen bonding constraints were to be invoked to optimize the suggested interactions defining the binding mode of the sugar moieties determined in the BR96-LeY complex. In general, structures of free and antigen bound antibodies demonstrate the flexibility of the antibody combining site and provide an example of induced fit as a mechanism for antibody-antigen recognition (35). Transitional differences are in fact observed for CDR1L, CDR3L and CDR2H of BR96 comparing ligated and unligated structures (32). Dynamic transitions within CDRs of unligtaed antibody forms have been suggested to be irrelevant to explain such induced fit binding mode geometry's (35). We observe that using homologous templates of antibodies that are co-complexed account for the observed transitions in CDR2L and CDR2H for BR96. Interestingly, CDR3H shows no difference in conformation between ligated and unligated BR96 (32). This might be because of the turn type and stabilizing effects contributed by Trp 100a interacting with a backbone carbonyl oxygen (32).

Placement of the Lewis Y tetrasaccharide core antigen in the B3 binding site.

Suggested hydrogen bonding sites between BR96 and the tetrasaccharide core structure of LeY (29) are observed to be highly conserved between BR96 and B3 (Figure 1). Suggested contacts are based upon heavy atom distances measured in the BR96 structure (29). Further analysis of these distances indicate that some of the suggested hydrogen bonding schemes are far from a geometry to form hydrogen bonds when considering the orienation of added hydrogens on donor - acceptor pairs. To identify the most likely set, the complexed BR96 based B3 structure was energy optimized with and without suggested hydrogen bonds as restraints followed by 100 ps of molecular dynamics. In a first set of calculations, no restraints were invoked (figure 2a). In this calculation Tyr H35 forms a hydrogen bond with OH-6^b, Ser

L27E forms a hydrogen bond with OH-4[d] and the backbone amide group of Ala H100 interacts with OH-4[c].

In a restrained calculation, the backbone amide group of Ala H100 was forced to form a potential hydrogen bond with OH-4[c], along with a hydrogen bond between His L27D and OH-3[b], and between O7[a] and the backbone NH group of Tyr H33. In this placement, the backbone amide of Leu H96 also interacts with OH-2[c]. Intermolecular energy calculations between LeY and B3 indicate that the confiiguration in Figure 2b is 3 Kcal more stable than that of Figure 2a. Imposition of the constraints in Figure 2b results in an RMS of 0.5 Å in the Cα positions relative to the conformation in Figure 2a. This analysis indicates that restraints are needed to fully realize idealized hydrogen bonding geometry's, but the imposition of these restraints does not alter significantly the overall conformation of the antibody template.

Peptide placement within the B3 combining site

In the placement of the B3 reactive putative peptide sequence APWLY, we made use of the program LUDI to identify compounds that potentially interact with the B3 combining site. Over 260 fragments were identified for the model, with the largest radius of interaction, with most redundant for the same set of potential hydrogen bond donors or acceptors on B3. In evaluating the fragments we compared fragments identified by LUDI relative to the APWLY sequence such that the fragments could occupy non-redundant sites and be spatially far enough from each other to accommodate the peptide backbone. In Figure 3 the placement of representative LUDI fragments is shown relative to their positions with each other within the B3 binding site of the respective models. LUDI found that a Trp like residue forms a hydrogen bond with the backbone carbonyl oxygen of Trp H98, that a lipophilic residue representative of a Leu side chain is bounded by residues Val L94, His L27D, and Ala H58 another lipophilic residue representative of an Ala and Pro side chain is bounded by Ala H97, (Figure 3).

The APWLY sequence was then modeled such that the corresponding Trp, Pro, Leu and Ala residues occupied relative positions as the identified LUDI fragments (Figure 4). In affect one wants to "stitch" the fragments together to form a peptide. We modeled the peptides two ways. The first, was to use individual amino acid fragments oriented with their side chains superimposed on the LUDI identified side chain types. The individual fragments were then restrained to form concomitant backbone geometry's

and conformations. As expected, such an approach resulted in highly strained conformations. Alternatively, a peptide was built and the phi, psi angles rotated until the respective side chains were in close proximity. The positioned peptide fragment-B3 complex was then energy optimized with a restrained dynamics calculation. After this dynamics run, the complex was again energy optimized to convergence without the imposition of constraints. Deviation of the backbone conformation of the peptide-B3 complex relative to the respective LeY-B3 complex was found to be only 0.29 A. This indicates that the placement of the peptide within the antibody combining site did not dramatically alter the overall conformation of either B3 structures.

While the LUDI search provided a favorable geometry for peptide side chain placement, the final placement of the peptide side chains within the antibody combining site relative to the LUDI positioned fragments were different (Figure 4). Several different starting geometry's for the peptide placement in the BR96 model were tested. Intermolecular interaction calculations indicate that the majority of the peptide binding comes from dispersion interactions. Five potential hydrogen bonds were found for the most stable of the models (Figure 5). One involves the N7 of Trp interacting with the backbone carbonyl group of Trp H98, the carbonyl backbone of Trp interacting with His L27D, the Tyr side chain hydroxyl group interacting with hydroxyl group of Ser H55, the backbone carbonyl group of Ala interacting with Asn H52A, and Tyr H33 side chain interacting with the carbonyl backbone of Leu, whose hydrophobic side chain being further stabilized by dispersion interactions with Val L94. We have further constrained the model peptide to form a beta turn inwhich a hydrogen bond is potentially formed between Tyr amide and Pro carbonyl groups.

In this positioning we observed that the Ala-Pro residues of the peptide occupied a similar position as the LeY GlcNAc residue. This positioning indicates that the proline residue mimics the spatial position of the glucose unit of GlcNAc, while the Ala methyl group is positioned similarly as the terminal methyl group of GlcNAc's N- acetyl (Figure 6). The Trp residue occupies a volume associated with the cFuc residue, and the Leu residue occupying the volume and the hydrophobic interaction of bGal. The Tyr residue occupies a position not associated with the LeY binding to B3.

Discussion

We have developed peptide mimics of carbohydrates that induce humoral immune responses reactive with bacteria, virus and tumor cells (20-22,36). This approach provides one more strategy that makes use of general immunological principles. In particular, peptides that mimic carbohydrates might be used to augment naturally available immunoglobulins to tumor antigens or induce memory responses to carbohydrates in a combined peptide/carbohydrate-conjugate vaccine approach for immunotherapy. Carbohydrate antigens by themselves are poorly immunogenic, difficult to synthesize and are important targets for immune attack. As demonstrated in our previous studies (19-22), appropriately constructed peptides may indeed be able to augment immunogenicity against carbohydrate antigens. The approaches to identifying these peptides include the use of phage combinatorial libraries for construction of a great range of peptides which are selected using carbohydrate reactive antibodies. From a molecular perspective, screening a phage display peptide library might identify a population of peptides reactive only with the isolating antibody. These peptides might, nevertheless, mimic salient features of a carbohydrate antigen reactive with a parallel set of carbohydrate specific antibodies. Modeling of isolated peptide and peptide monoclonal antibody conformations might suggest sites of amino acid changes in the peptides for augmenting antibody specificity or designing peptides that induce a broad range of carbohydrate reactive antibody subsets.

As a model system, we examined the molecular basis for reactivity of a peptide that mimics the tetrasaccharide core of the Lewis Y antigen, isolated from phage display screening (18). Based upon the crystal structure of the BR96-LeY tetrasaccharide complex and the relative sequence similarities between anti-LeY antibodies (Figure 1), it is apparent that the MAb binding groove of LeY specific MAbs is sufficiently large to bind four monosaccharide units of the LeY determinant (Figure 2) and fit a putative peptide surrogate that effectively mimics LeY binding. The similarities among the anti-LeY antibodies provides a restraint on the binding site configuration for LeY. The modeling of B3 reflects this point. The imposed binding mode restraint provided from the BR96-cocomplex provides limits as to the conformational transitions the CDR domains will undergo. It is well known that most changes in CDRs occur in the first 100 picoseconds of molecular dynamics runs (34). However, the imposition of long dynamics run on unligated or uncomplexed structures do not represent potential binding mode geometry's

for an antibody (35). Structural analysis of unligated BR96 indicates that L1, L2 and H2 loops undergo transitions upon antibody binding, while H3 does not (32). In our comparison we show that the conformational transitions observed for L1, L2 and H2 are those observed in other ligated antibodies. In effect this observation validates a rationale of using antibody templates that have undergone antigen binding to appropriately model a transition state conformation for a ligated antibody form. In our modeling of B3, we so heavily constrain the movement of the B3 template backbone that only the side chains adjust. In this way we are preserving the crystal structure determined binding mode geometry for BR96 as adopted by B3. Subsequently, for antibodies, it is not at all clear why MD calculations should ever be performed on unligated structures when one is interested in ligated conformations.

Structural studies on Lewis antigens have generally substantiated that conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles. Molecular dynamics calculations on Lewis antigen structure prototypes indicate the lack of spontaneous conformational transitions to other minima during the simulations, suggesting that these oligosaccharides maintain well-defined conformations with relatively long lifetimes (28, 37). These results further indicate that hard sphere or rigid-geometry calculations, albeit in the absence of solvent, provides a good picture of the steric repulsion that modulate the conformational properties of the Lewis antigens. Subsequently the notion of utilizing a structure based drug design approach (31) to determine a possible binding mode of a carbohydrate surrogate peptide offers a novel approach to confirm the ability of certain peptide residues to participate in contacting a receptor.

The search of a fragment library for possible compounds that would fit within the B3 combining site, provides a guide to position the side chains of the putative peptide sequence APWLY to effectively compete with the LeY antigen (Figure 3). The binding mode of the peptide did not faithfully mimic the LeY antigen in contacting all the same functional groups on B3, but binds in a fashion that provides for at least steric competition between the peptide and the LeY structure (Figure 5). We observe that the peptide might be availed to form a beta turn in the binding site. This conformation lends itself to the Tyr residue of the peptide to potentially interact with several residues in CDR2 of the heavy chain of B3 which include Asp H53, Ser H52, Ser H55 or Ser H56. These residues are different with respect to BR55-2 which does

not effectively bind the APWLYGPA peptide (unpublished observations). It is noted that the positioning of the peptide within the B3 combining site is strictly a model and awaits confirmation from crystallographic studies on related anti-LeY reactive antibody-peptide complexes. Nevertheless, the model does suggest that peptides that contain the APWLY motif should bind to B3, effectively mimicking the LeY antigen as observed experimentally. The extent of potential fragments that we have found to interact with the B3 combining site indicates that there may still be many ways for peptides with differing sequences to interact with B3 in spite that only one peptide sequence was identified in the phage screening (18). In related studies, we have identified many peptides reactive with BR55-2 from phage display screening with BR55-2 (unpublished). One peptide within this set displays an inverted WPYL sequence compared with PWLY. While it is likely that peptides identified by screening phage libraries might only be reactive with an isolating antibody, the approach described here might help to identify potential peptides for immunization studies from the many peptide sequences identified from phage display screening to induce a broadly reactive antibody subset. In particular, further structural studies of histo-blood group related antigen binding to specific antibodies can provide information relevant to vaccine design strategies and improved immunotherapeutics for a variety of human cancers over-expressing these and related carbohydrate determinants.

Acknowledgments

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Figure Legends

Figure 1: Sequence alignment of variable region Light and Heavy chain s of B3 with crystal template structures. Sequences for BR96, TE33, BV04, and B13i2 are from deposited PDB files in the Brookhaven repository (38). Sequence for B3 is from (24) and BR55-2 from (28). Numbering corresponds to that of the BR96 crystal structure. Respective CDRs are underlined for each chain. LeY contact residues that are conserved between the anti-LeY antibodies BR96, and B3 are in Bold face on the BR96 sequence.

Figure 2: Hydrogen bond interaction schemes of B3 with the LeY tetrasaccharide core under different constraint conditions. The position of LeY is that observed in the BR96 crystal structure. The a,b,c,d designations refer to the βDGlcNac, βDGal, αLFuc(1→3) and αLFuc(1→2) units, respectively. Figures 2a-2b are interaction schemes for the BR96 based model. Intermolecular interaction energies are -73 Kcal (Figure 2a), and -76 Kcal (Fiure 2b). Drawings were made with LIGPLOT (39).

Figure 3. Selected LUDI fragments identified as representative amino acid side chains comprising the APWLY motif. B3 is colored yellow in the BR96 based model. Selected fragments represent Ala, Pro, Trp, and Leu side chains.

Figure 4. Fitting of the putative peptide sequence onto the LUDI defined fragments. The conformation of the APWLY peptide (colored cyan) is energy optimized binding mode in the B3 - BR96 template

derived combining site. The Pro like fragment is colored magenta, the Trp like fragment is colored green and the Leu like and Ala liphophilic groups are colored orange.

Figure 5. Low energy placment of the APWLY motif within the B3 combining site. The APWLY sequence is colored green, highlighting nitrogens and oxygens colored blue and red respectively, with hydrogens colored white. The B3 antibody is colored orange. The N terminus of the APWLY peptide is colored magenta and its C terminus is colored yellow. Both termini are pointing outward from the combining, simulating a required orientation of the ends for extended residues. Hydrogen bonds are illustrated to respective B3 amino acids in the combining site.

Figure 6. Overlap of the putative peptide with the LeY tetrasaccharide core. The peptide and the LeY structure are those of the binding mode conformation within the BR96 template derived combining site. In this orientation of the peptide within the site the Ala and Pro residues of the putative peptide sequence are observed to be spatially similar to the GlcNAc residue of the LeY structure.

LIGHT CHAIN

```
                1                   10                  20                27 A B  C D E 28  30                  40
BR96    D V L M T Q I P V S L P V S L G D Q A S I S C R S S Q I I V H N N G N T Y L E W Y L Q K P G
B3      - - - - - - S - L - - - - - - - - - - - - - - - - - S - - - S - - - - - - - - - - - - - -
BR55    - - - - - - T - L - - - - - - - - - - - - - - - - - S - - - S - - - - - - - - - - - - - -
TE33    - - - - - - T - L - - - - - - - - - - - - - - - - K - - - S - - - S S - - - - F - - - - - -
BVO4    - - V - - - T - L - - - - - - - - - - - - - - - - - S L - - S - - - - - - - H - - - - - - -

                                50                  60                  70                  80
BR96    Q S P Q L L I Y K V S N R F S G V P D R F S G S G S G T D F T L K I S R V E A E D L G V Y Y
BR3     - - - K - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
BR55    - - - K - - - S - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
TE33    - - - K - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
BVO4    - - - K - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - F

            90
BR96    C F Q G S H V P F T F G S G T K L E I
B3      - - - - - - - - - - - - - - - - - -
BR55    - - - - - - - - - - - - - - - - - -
TE33    - - - - - - I - - - - - - - - - - -
BVO4    - S - S T - - - L - - - A - - - - - L
```

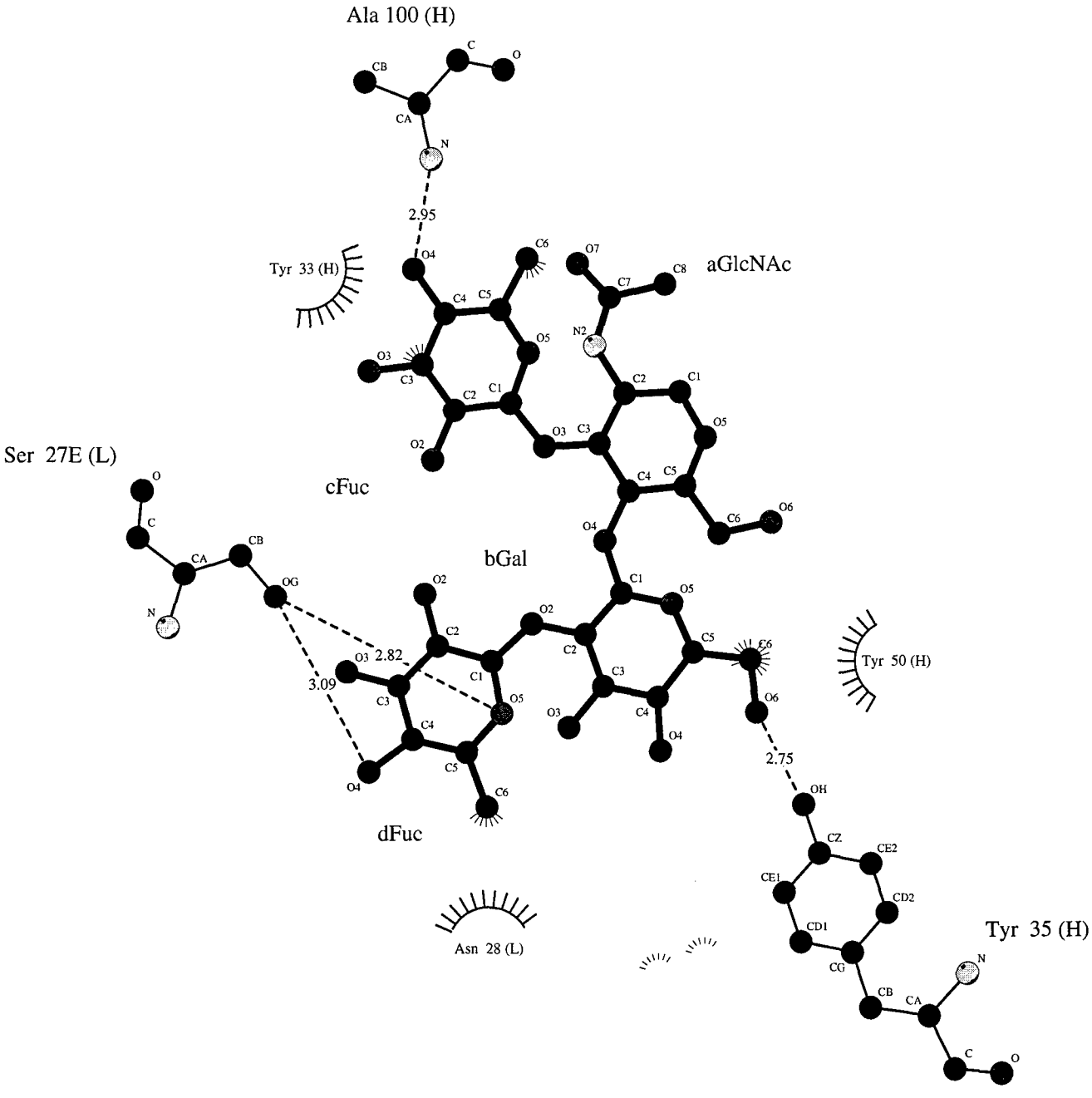
HEAVY CHAIN

```
                1                   10                  20                  30                  40
BR96    E V N L V E S G G G L V Q P G G S L K V S C V T S G F T F S D Y Y M Y W V R Q T P E K R L E
B3      D - K - - - - - - - - - - - - - - - - L - - A - - - - - - - - - - - - - - - - - - - - - -
BR55    - - K - - - - - - - - - - - - - - - - L - - A - - - - - - - - - - - - - - - - - - - - - -
B13i2   - - Q - - - - - - - - - K - - - - - - L - - A A - - - - - - R C A - S - - - - - - - - - -

                50  52 a 53              60                  70                  80  82 a b c 83
BR96    W V A Y I S Q G G D I T D Y P D T V K G R F T I S R D N A K N S L Y L Q M S R L K S E D T A
B3      - - - - - - N D D S S A A - S - - - - - - - - - - - - N - - R - T - - - - - - - - - - - -
BR55    - - - - - - N - - G S S H - V - S - - - - - - - - - - - - - - - T - - - - - - - - R - - - - -
B13i2   - - - G - - S - - S Y - F - - - - - - - - - - - I - - - N - - R - T - S - - - - - - R - - - - -

            90                  100 a b 101
BR96        M Y Y C A R G L D D G A W F A Y W G Q G T L
B3          I - S - - - - - A W - - - - - - - - - - - -
BR55        - - H - - - - M - Y - - - - - - - - - - - -
B13i2       I - H - T - Y S S - P F Y - D - - - - - - -
```

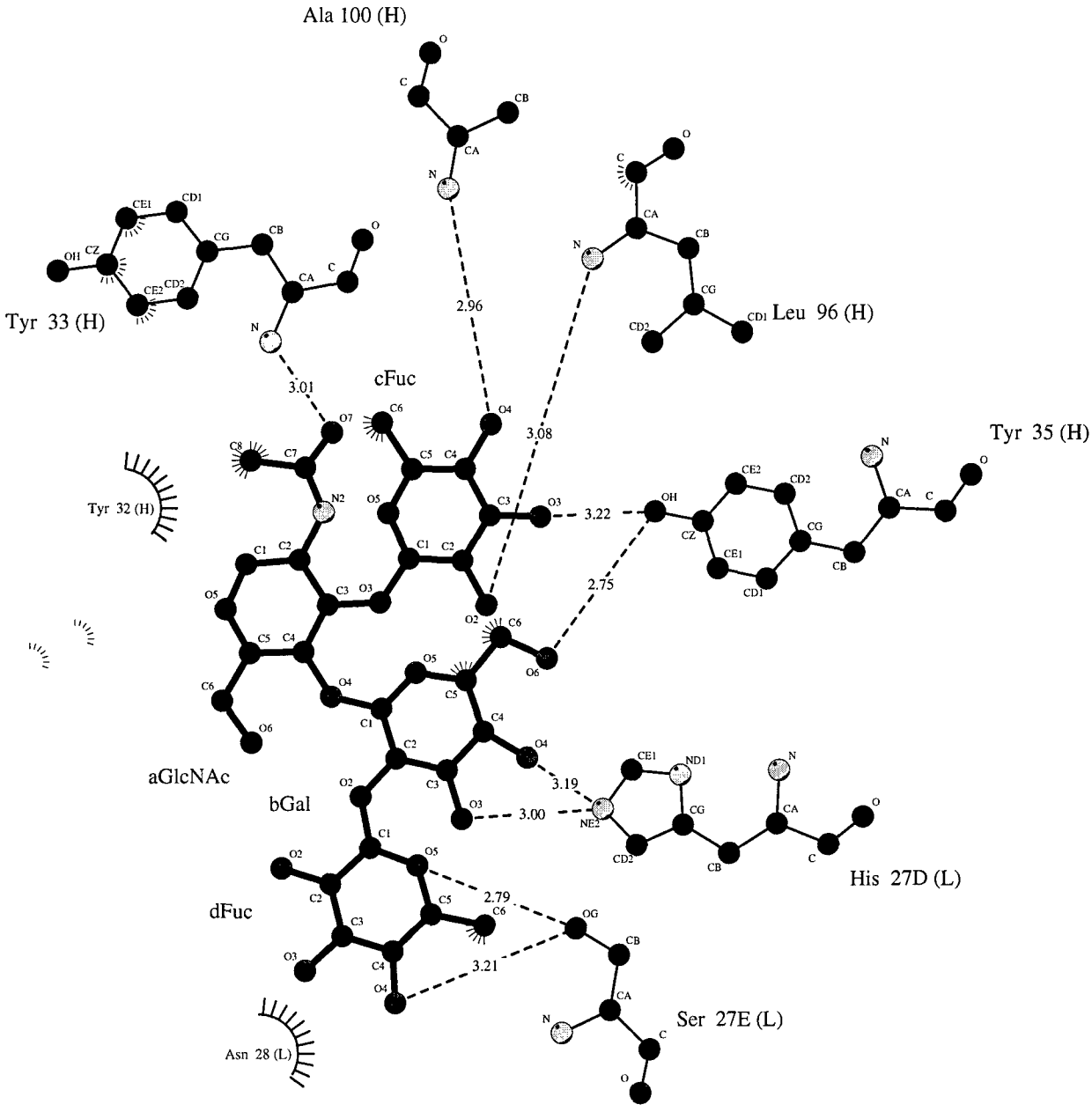
Figure 1



Key

- Ligand bond
- Non-ligand bond
- Hydrogen bond and its length
- His 53 — Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

Figure 2a



Key

- Ligand bond
- Non-ligand bond
- 3.0 Hydrogen bond and its length
- His 53 Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

Figure 2b

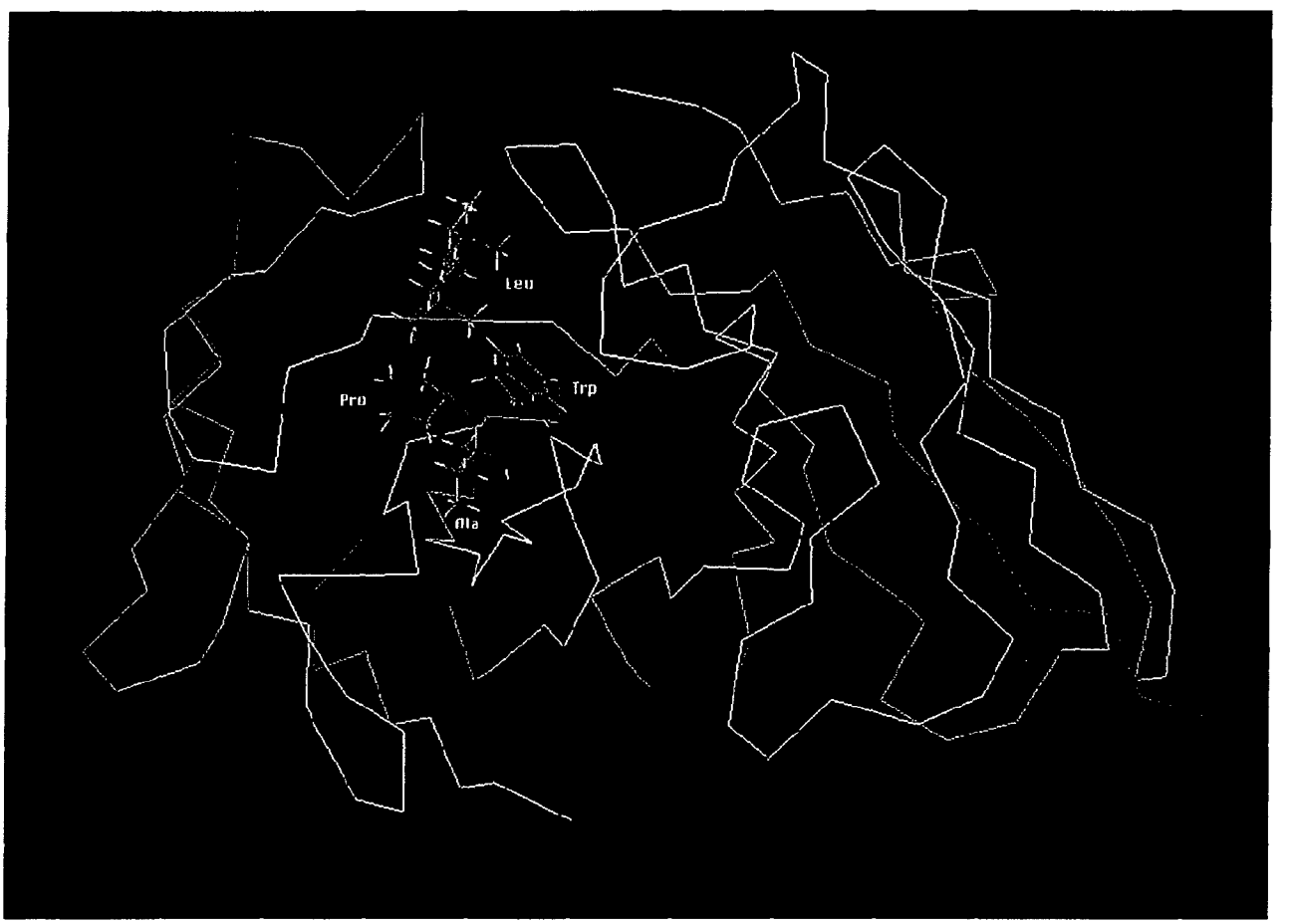


Figure 3

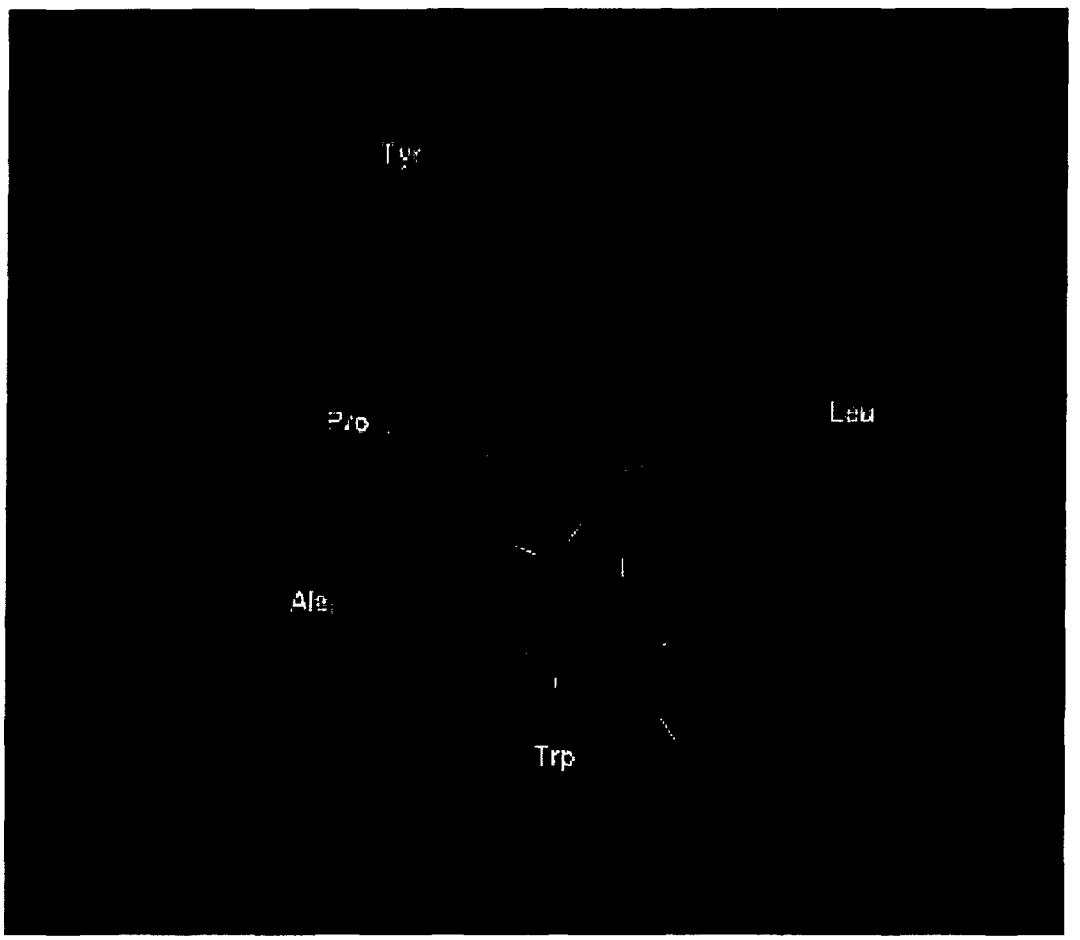


Figure 4

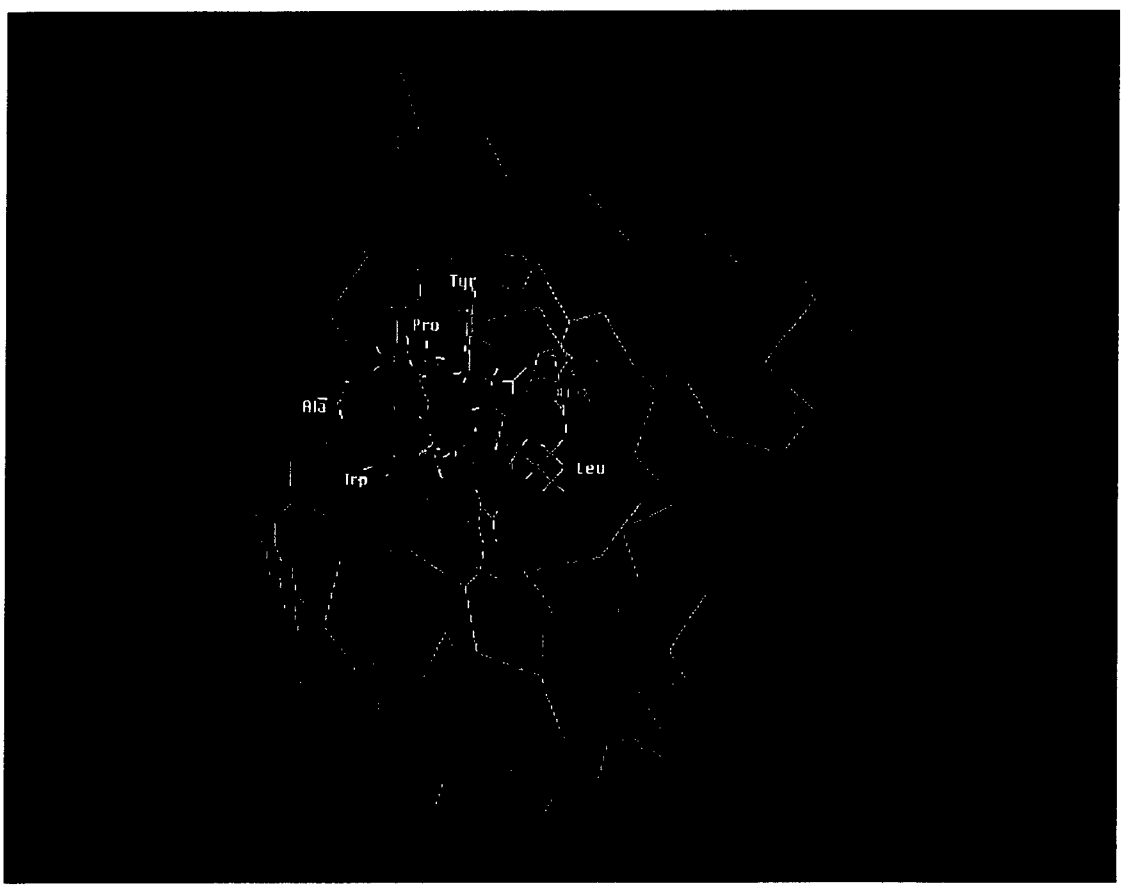


Figure 5

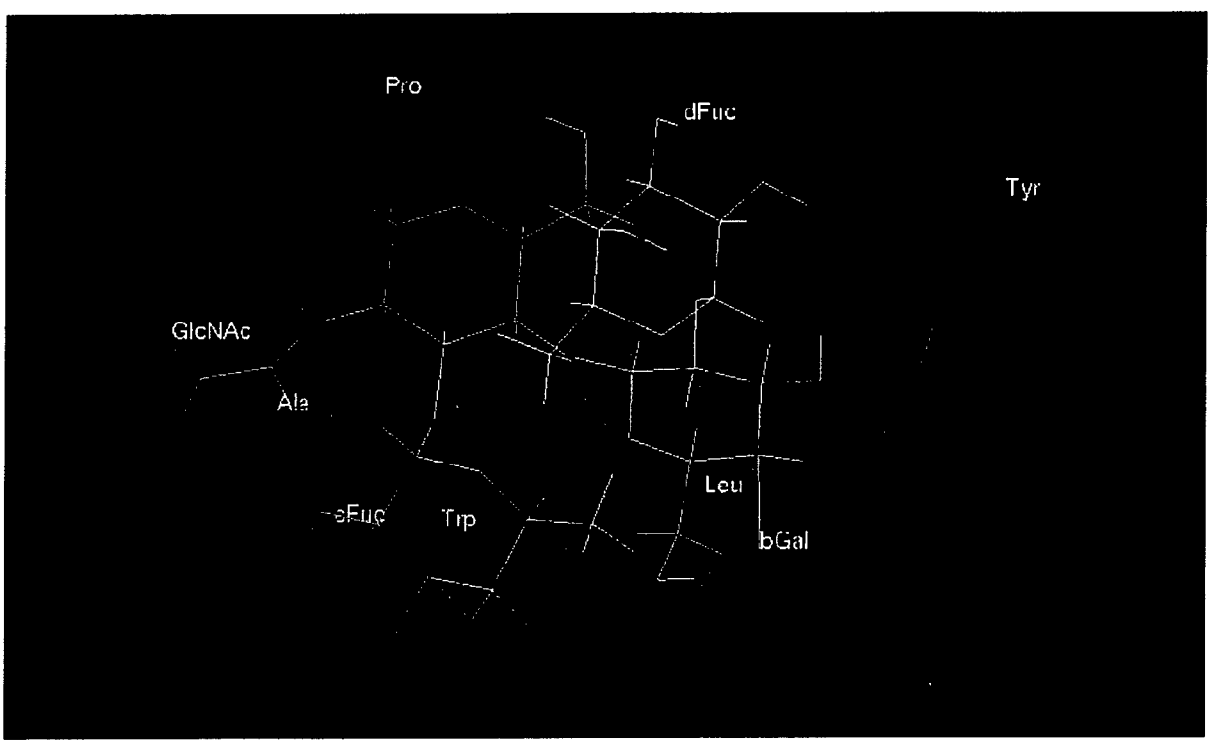


Figure 6

HYBRIDOMA
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Peptide Mimicry of Adenocarcinoma-Associated Carbohydrate Antigens

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ABSTRACT

Carbohydrate antigens have been identified as significant antigens in many human tumors either by analyzing antibodies in patients' sera or by using monoclonal antibodies of either mouse or human origin. Three carbohydrate epitopes present on cancer-associated mucins [sialyl-Lewis A (SLA), sialyl-Lewis X (SLX), and siayl-Tn (STn)] may have functional significance in metastasis. Subsequently, these antigens are considered as targets for active specific immunotherapy. Carbohydrates, as T-cell-independent antigens, often elicit diminished immune responses. To overcome this drawback, carbohydrates are typically coupled to protein carriers to elicit immunoglobulin G (IgG) responses as opposed to low-affinity IgM responses, which oftentimes accompanies carbohydrate-based immunizations. In addition, some complex carbohydrates are difficult to synthesize. This latter aspect is further magnified if one considers that clustering of epitopes on neoglycoproteins must be emulated in the synthesis process, leading to multiple presentation or tandem repeats of the synthetic carbohydrate immunogen. Here, we examine the hypothesis that peptides that mimic carbohydrates might be developed to induce immune responses that target and mediate the killing of tumor cells, particularly breast cancer cells in an adjuvant-type setting. We have found that carbohydrate-mimicking peptides retain carbohydrate-like conformations, inducing anti-carbohydrate immune responses against breast tumor cells and mediating their killing by a complement-dependent mechanism.

INTRODUCTION

CELL-SURFACE CARBOHYDRATES undergo dramatic changes in cancer, playing a crucial role in cell–cell communication, cell growth and differentiation.[1] Aberrant glycosylation of tumors relative to their normal counterparts, represents a phenotypic feature associated with different human malignancies.[1,2] This phenomenon is demonstrated repeatedly at frequencies higher than those of oncogenes and suppressor genes in various tumors. Aberrant glycosylation influences tumor progression because cells acquire competence for metastasis and faster clonal growth via newly synthesized carbohydrate structures.

Several types of altered glycosylation have been described in human carcinomas and are prevalent in mammary adenocarcinoma (some common ones illustrated in Fig. 1): (i) enhanced expression of GlcNAcβ1 → 6Manβ1 → 6 units appear to correlate with progression of human mammary carcinoma[3,4]; (ii) the T and Tn structures, Galβ1 → 3GalNAcα and GalNAcα, respectively, are powerful histologic markers in diagnosis and prognosis, occurring as surface antigens on most primary human breast carcinomas and their metastases, and are able to elicit both humoral and cell-mediated immunity[5–7]; (iii) the Thomsen Friedenreich (TF) antigen is an important disaccharide panadenocarcinoma antigen that targets antibodies for tumor killing[8]; (iv) human breast carcinomas express the histo-blood group antigen H (type 2) Fucα1 → 2Galβ1 → 4Galβ1 on the globoside backbone[9]; (v) sialylated derivatives of type 1 and 2 Lewis antigens such as sialyl-Le^a (SLA) NeuAcα2 → 3Galβ1 → 3(Fucα1 → 4) GlcNAcβ1 → 3Galβ1 → 4 Glcβ1 → R and sialyl-LeX (SLX) NeuAcα2 → 3Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → R are tu-

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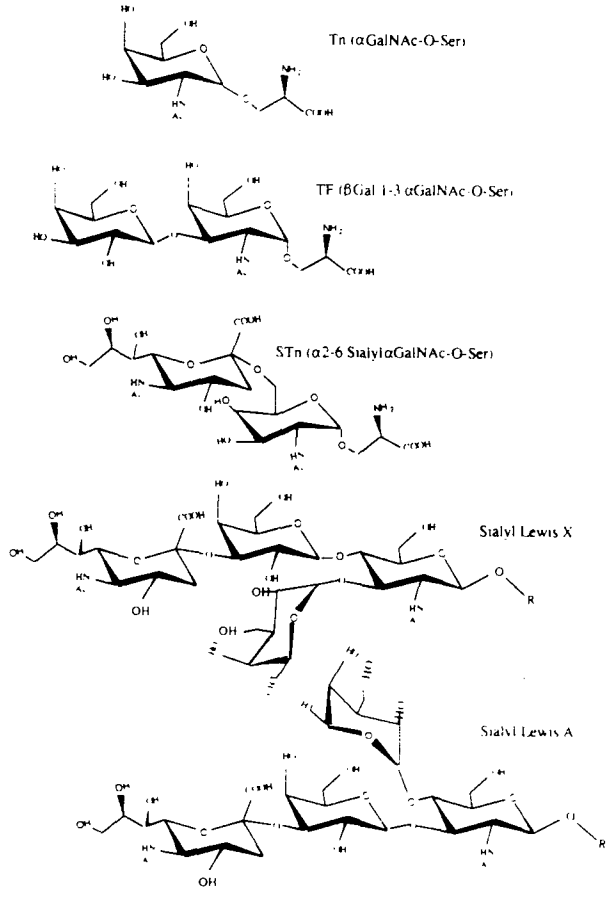

FIG. 1. Common adenocarcinoma-associated carbohydrate structures.

mor-associated gangliosides and are observed more frequently than their respective nonsialylated forms in breast adenocarcinomas[10]; (vi) the accumulation of α-fucosylated derivatives of lactoseries type 1 and 2 structures such as Lewis Y (LeY), Fucα1 $\rightarrow$ 2Galβ1 $\rightarrow$ 4(Fucα1 $\rightarrow$ 3)GlcNAcβ1 $\rightarrow$ 3Galβ1 $\rightarrow$ 4 Glcβ1 $\rightarrow$ R, H-2 and Lewis b (Le^b) is closely associated with adenocarcinoma development and correlates inversely with patient survival with primary lung adenocarcinoma.[11-13]

Epidemiological studies have shown that patients expressing SLA, SLX, sialyl-Tn (STn), Lewis X (LeX), and LeY structures on their primary tumors have markedly lowered survival rates. STn formulations have been shown to induce immune responses to breast cancer and formulations are in clinical trails.[13,14] Immune responses to some Lewis antigens is expected to be weaker in humans compared to other mammals. From a vaccine perspective, responses to carbohydrates as tumor-associated antigens (TAAs) are notorious in eliciting diminished immune responses, with predominately IgM antibodies displaying relatively low affinity for carbohydrates.[15,16] Immune responses can be enhanced however by coupling carbohydrates to carrier proteins and adjuvants which is perceived to recruit T-cell help.[15,17-26]

Although these results show promise for active specific immunotherapy with carbohydrate formulations, they point out the limitations of this approach. These include: difficulty in antigen purification or synthesis, the utility of carbohydrate carrier-protein coupling strategies that might prove to be impractical for broad application, and the lack of persistent high-titer cytotoxic antibodies in many patients. These difficulties might be further magnified by considering that neoglycoconjugates in which the carbohydrate structures are clustered together might make better immunogens.[8,27] It is argued that such structures would more closely resemble the arrangement of carbohydrate epitopes on the cell surface—either glycolipid aggregates, multiantennary N-linked chains, or clustered O-linked oligosaccharides on mucins. Despite this widely held view, there is little experimental support for this concept. The most convincing evidence for this concept is from examining the specificity of monoclonal antibodies (mAbs) raised to cells or other sources. For example, in a recent study, it was shown that a clustered neoglycoprotein antigen consisting of STn substituted on a triserine peptide, which in turn is coupled to keyhole limpet hemocyanin (KLH), gave an antibody response in mice that recognized clustered epitopes and natural forms of STn.[27] Despite these promising results, synthetic routes for developing clustered carbohydrate epitopes is not straightforward.

Carbohydrates that influence the metastatic potential of tumor cells are not restricted to just tumors. Adhesion of pathogens and toxins to host cell plasma membrane is mediated by glycoconjugates. Subsequently, the distribution of carbohydrates contributes to the cross-reactivity of antibodies for common saccharide subunits among bacteria, viruses, and tumor cells. Immunochemical studies of sialylated lipo-oligosaccharides (LOS) of the Gram-negative bacteria *Neisseria gonorrhoeae* and *N. meningitidis* suggest that structural characteristics of these LOS might relate to their roles during pathogenesis. The carbohydrate moieties of the LOS of pathogenic *Neisseria* mimic carbohydrates present in glycosphingolipids of human cells.[28] Such studies indicate that the pathophysiology of processes such as infection and neoplasia are profoundly affected by the same or similar carbohydrate forms.

We have shown that antibodies induced by a peptide mimic of the major C polysaccharide (MCP) of *N. meningitidis* can induce antibodies that react with MCP and protect mice from lethal challenge with *N. meningitidis*.[29] MCP is a polymer of $\alpha(2 \rightarrow 9)$ sialic acid. Structural analysis of MCP suggests that its conformation is similar to subunits associated with carbohydrate forms associated with breast tumors. Here, we show that peptides that mimic carbohydrate subunits can induce humoral responses that cross-react with representative synthetic carbohydrate probes associated with breast tumors, and with tumor cells, mediating complement-dependent cytotoxicity (CDC) of human breast cell lines.

MATERIALS AND METHODS

Preparation of peptide–proteosome complex

Peptides were synthesized with the addition of a tripeptide YGG spacer, and a cysteine at the N terminus conjugated to a lauroyl group (Bio-Synthesis, Lewisville, TX). The meningococcal outer membrane proteins or proteosomes were prepared and complexed to the lauroyl-C-YGG-peptides as described by Lowell *et al.*[30,31] in a 1 mg:1 mg ratio, combining the com-

ponents in the presence of detergent. The detergent was removed by extensive dialysis.[30,31] Proteosomes were prepared from outer membrane complex vesicles from group B meningococci, strain M99, by ultracentrifugation and soluble in detergent buffer.[32] The lauroyl group allows for hydrophobic complexing of the peptide to the proteosomes whereas the cysteine at the N terminus appears essential for immunogenicity, apparently cross-linking multiple epitopes.[30,31]

Preparation of antibodies against carbohydrate-mimicking peptides

For generation of polyclonal sera BALB/c mice ($n = 4$ per group) 4–6 weeks of age, were immunized i.p. on a weekly basis for 3 weeks with 50 μg of peptide–proteosome complex. Sera was collected within 7 and 14 days after the last immunization and analyzed for binding against synthetic carbohydrate probes by enzyme-linked immunosorbent assay (ELISA) and against breast cells using fluorescence-activated cell-sorting (FACS) analysis.

ELISA assays

Solid-phase ELISA was performed to assess the binding activity of sera induced by the carbohydrate-mimicking peptides to a variety of carbohydrate synthetic probes incorporated into polyacrylamide matrix, (creating 30-kDa multivalent polymers, purchased from Glycotech). Immunlon 2 plates were coated with the Fucα1-4GlcNAc, LeY, Galβ1-3Gal, Galβ1-3GalNAc, Sialyl-Lea, Lea, Sialyl-Lex, Lex, Lex pentasaccharide, and Leb-hexasaccharide overnight at 4°C. The plates were blocked with 0.5% fetal calf serum (FCS)/0.2% Tween, 200 μl/well, 37°C, for 1 h. Serial dilutions of the respective antisera were added and resolved with 100 μl/well of 1:10,000 × goat anti-mouse immunoglobulin (IgG) isotype-matched horse radish peroxidase (HRP) (Sigma) diluted in blocking buffer and incubated at 37°C for 1 h. OD_{450nm} was read using a Dynatech MR5000 ELISA reader.

FACS analysis

For the preparation of cells, 10 ml of FACS buffer was added and the cells were washed, scraped, and transferred to 15-ml centrifuge tubes. Viable cells were counted by trypan blue. Cells were diluted to 2×10^6/ml and 100 μl used for each sample. Primary sera (10 μl) was added to the sample tubes and incubated on ice for 30 min, washed twice with 1 ml of FACS buffer, and centrifuged for 2 min at 1,500 rpm. A total of 10 μl of fluoroisothiocyanate (FITC) Ab [goat anti-mouse IgG FITC-labeled (Sigma) diluted 1:20 with phosphate-buffered saline (PBS)] was added to the sample and incubated on ice for 30 min and again washed twice with FACS buffer. Cells were fixed using 2% paraformaldehyde, followed by FACS measurement.

Complement-dependent cell cytotoxicity

Briefly, sera was tested for its ability to bind to tumor lines and modulate complement-dependent cell cytotoxicity (CDC). Ten microliters of each cell line (4×10^4 cells/ml) were added to duplicate wells of a microtiterplates, to which was added 20 μl of serially diluted sera, and incubated for 45 min on ice. Rabbit complement (20 μl, 1:2) was added and allowed to incubate for 4 h at 37°C. The medium was discarded and 50 μl of methanol fix was added and allowed to incubate for 10 min. This procedure was repeated once again for 5 min. The number of viable cells was determined by Giemsa stainning. A 2% Giemsa stain in methanol was added and allowed to fix for 25 min and then rinsed in distilled water. Plates were counted under a light microscope. The percent of cytotoxicity (PC) was calculated with the formula: PC = [1 − (number of cells in well treated with antibody and complement/number of cells in well treated with medium only)] × 100. Control wells did not contain antisera.

RESULTS

The induction of anti-carbohydrate immune responses by peptides

Recently, we have shown that the peptide, CARIYYRYDGTAY [derived from an anti-idiotypic antibody that mimics the major C polysaccharide (MCP) of *N. meningitis*] when complexed to proteosomes induces an anti-MCP antibody response in BALB/c mice that is protective in nature.[29] The major Ig fraction upon immunization was found to be IgM, with IgG coming up later.[29] This peptide bears some homology with other peptides that mimic carbohydrates involving a Planar-X-Planar sequence motif (Table 1). In particular, Hoess *et al.*[33] have identified an 8-amino-acid peptide expressed on phage capable of inhibiting LeY carbohydrate–antibody binding and consisting of the sequence tract PWLY. The sequence similarities among the putative motifs suggest that antibodies raised to this peptide set might cross-react with similar subunits expressed on what are otherwise dissimilar carbohydrate structures. For example, antibodies that recognize MCP might cross-react with the sialyic acid portions of SLX and SLA. It is also possible that anti-peptide sera reactive with MCP react with the tetrasaccharide core of LeY because the peptide motif that mimics LeY bears similarity with the peptide motif that mimics MCP (Table 1 and Fig. 1).

The possible structural similarities suggest that antisera raised to the peptide putative motifs might cross-react with a variety of subunits representative of Lewis antigens. The immunological presentation of the putative motifs (*i.e.*, short or

TABLE 1. SEQUENCE MOTIFS THAT MIMIC CARBOHYDRATE STRUCTURES

Peptide	*Carbohydrate*	*Structure*	*Reference*
YYPY (P1)	Mannose	Methyl-α-D-mannopyranoside	41, 42
WRY	Glucose	$\alpha(1 \rightarrow 4$glucose	
PWLY	Lewis Y	Fucα1 → 2Galβ1 → 4(Fucα1 → 3)GlcNAc	33
YYRYD (P2)	Group C Polysaccharide	$\alpha(2 \rightarrow 9)$sialic acid	29

longer peptides, presentation in a helix or β-bend) might mimic overlapping epitopes on otherwise different carbohydrate structures. To test this idea, BALB/c mice were immunized with peptides representative of the motif YYPYD (P1), YYRYD (P2), and the tract YYRGD (P3). To enhance their immunogenicity, the peptides were complexed to hydrophobic meningococcal group B outer membrane proteins, referred to as proteosomes. The peptides were synthesized with the addition of a 3-amino-acid spacer and a lauroyl group. The meningococcal outer membrane proteins or proteosomes were prepared and complexed to the lauroyl-C-YGG-peptides in a 1 mg:1 mg ratio.[29] Sera were collected 1 week after the last immunization and tested for reactivity against MCP and carbohydrate probes of Lewis antigens or their subunits.

For ELISA assays, plates were coated with synthetic carbohydrate probes and allowed to react with the respective antisera (Fig. 2). As a positive control, the monoclonal antibody BR55-2, which displays specificity for LeY, was used.[34,35] The monoclonal antibody ME361, which displays specificity for the GD2 ganglioside, was used as a negative control.[36] For BR55-2, selective binding was observed for LeY with some reactivity for the Galβ1 $\rightarrow$ 3GlcNAc structure representative of the TF antigen. We also observed binding with the related core structure Galβ1 $\rightarrow$ 4GlcNAc shared between LeY and LeX (data not shown). However, this core structure is apparently not seen by BR55-2 when present in the LeX-pentasaccharide. ME361 was not reactive with any of the synthetic probes. It was observed that the anti-peptide sera (1:200 titer) was reactive with respective carbohydrate probes above the FCS background binding, suggesting commonalties in recognition of functional groups among the subunits as indicated in Figure 1. Antisera to the YYRGD motif was consistently two-fold above the FCS background, followed by sera to the YYRYD motif displaying about 1 1/2 times above background. Sera generated against the YYPYD motif was observed to range between two- to six-fold above FCS reactivity. These data suggest that there is a large degree of overlap in the potential carbohydrate structures being recognized by the antisera, a result that was expected because the peptides mimic a wide range of individual carbohydrate subunits.

FACS analysis of tumor cells with anti-peptide sera

The extent of cross-reactivity of the sera among the synthetic probes suggests that the sera should bind to breast cells. The ability of the sera to bind to breast cells was evaluated by FACS analysis. We found that murine sera elicited against the peptides bind to the human breast line SKBR3, which expresses high a level of lactoseries structures (Table 2), but there was little binding to the normal human breast line HS578. Some cross-reactivity of the anti-P3 sera to NIH-3T3 cells was observed, but its specificity for SKBR3 was four-fold higher.

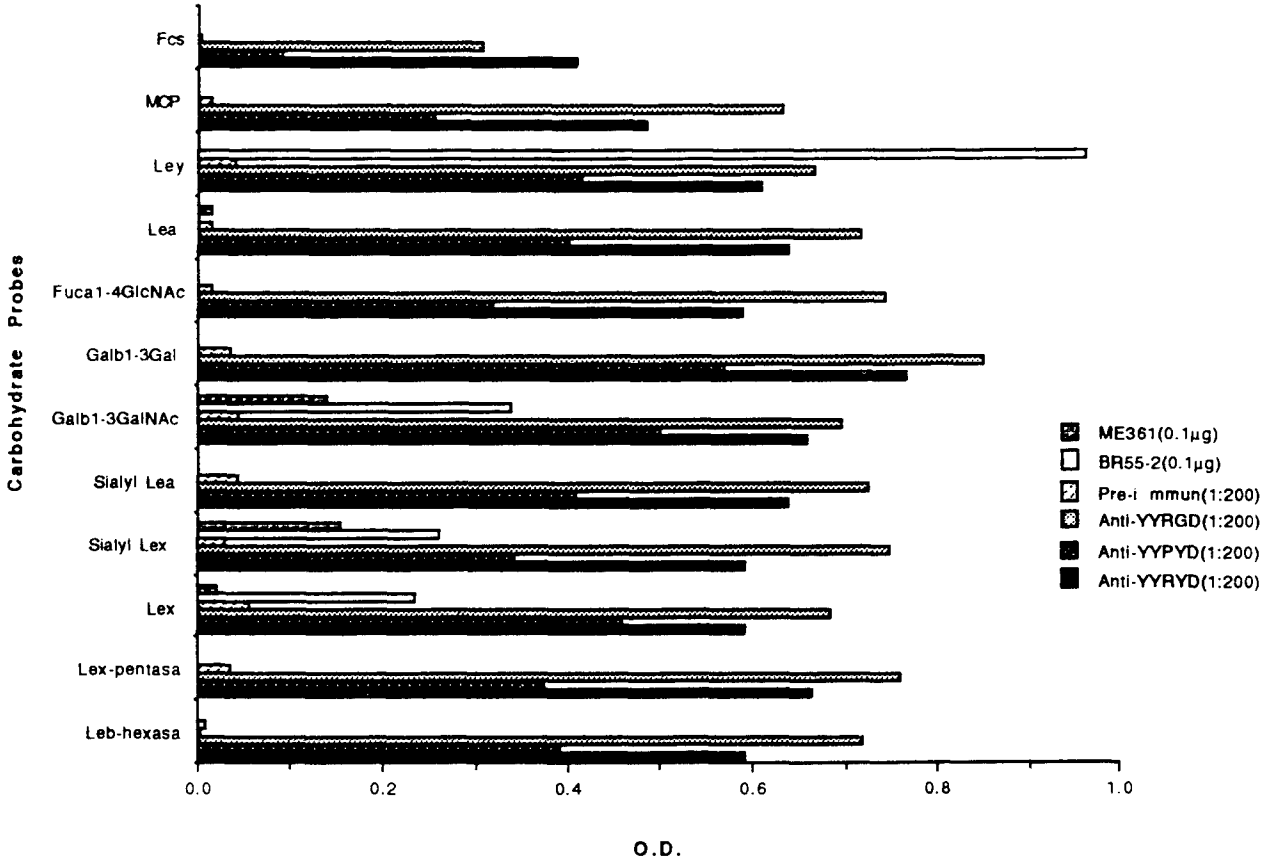

FIG. 2. ELISA profiles of synthetic carbohydrate probes with the anti-peptide sera.

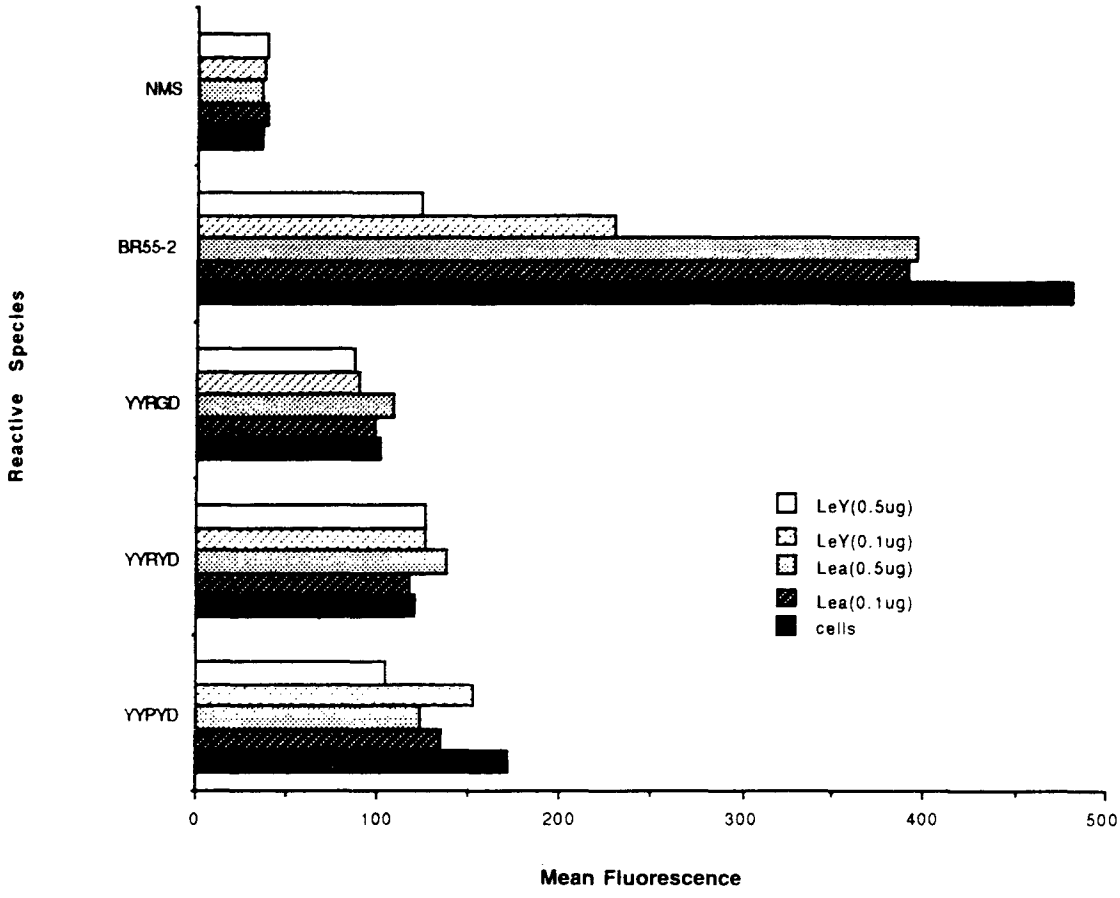

FIG. 3. Inhibition of binding of antisera to SKBR3 cells.

Some binding was also observed for the WM793 human melanoma cell line. For the melanoma cell line, some differences among the sera were displayed. These data suggest that the antisera for the most part recognize overly expressed carbohydrate antigens on human breast tumors, with diminished reactivity for normal breast and control cell lines.

We also examined the ability of synthetic probes to inhibit the binding of the sera to SKBR3 cells. Figure 3 summarizes one experiment in which the ability of LeY and Lea to inhibit the respective sera from binding to SKBR3 cells was assessed by FACS analysis. The positive control antibody BR55-2 is LeY inhibitable by over 50% within the concentration range used. Normal mouse serum (NMS) is near background levels, as observed previously (Table 2). Although the antisera to YYRYD and YYRGD motifs are not inhibitable at the concentrations used for LeY and Lea, antisera to the YYPYD motif is inhibitable by the LeY probe over the concentration used. These data suggest that the anti-YYPYD sera is sensitive to the LeY probe, similarly as the anti-LeY antibody BR55-2.

TABLE 2. BINDING OF THE ANTI-PEPTIDE SERA TO DIFFERENT CELLS AS MEASURED BY FACS

Cell line	*Anti P1 (YYPY)*	*Anti P2 (YYRYD)*	*Anti P3 (YYRGD)*
SKBR3	171.0	120.0	101.0
HS578 Bst (normal breast)	17.8	19.9	22.4
WM793	145.5	172.4	42.3
NIH-3T3 Murine fibroblasts	20.9	21.8	41.7

Representative FACS experiment. Final sera concentration was 1:50. Background fluorescence (mean fluorescence) associated with nonspecific mouse sera is 24.2, 25.2, and 23.7 for SKBR3, and NIH-3T3 cells, respectively. Background for the human melanoma line WM793 was, on average, 24.4.

Mediated cytotoxicity

We next examined the ability of the sera to mediate CDC of the SKBR3 human adenocarcinoma cell line. In Figure 4A we show that the anti-LeY antibody BR55-2 mediates CDC at high antibody concentrations, approaching 80% at 100 μg. The negative control antibody ME361 did not mediate CDC of SKBR3. In Figure 4B we show that the three lead anti-peptide sera mediate CDC of SKBR3 about the same, whereas normal mouse sera did not. This result was observed for the MCF7 line as well (data not shown). Although the antisera were observed to bind to the human melanoma WM793 line, as assessed by FACS (Table 2), we did not observe CDC killing of this cell line (data not shown). These data suggest that the peptides can induce immune responses that target tumor cells, mediating their killing *in vitro*. The relatively low titers for effective killing might be the result of the density of carbohydrate epitopes on the cell. More faithful peptide mimics of the respective mucin epitopes, clustered in a fashion to mimic the high carbohydrate density, might prove more effective in mediating killing.

DISCUSSION

Mucins are attracting great interest as potential targets for immunotherapy in the development of vaccines for cancers (*e.g.*, breast, pancreas, ovary, and others) because there is a 10-fold increase in the amount in adenocarcinomas, an alteration in expression where they become ubiquitous, and, due to al-

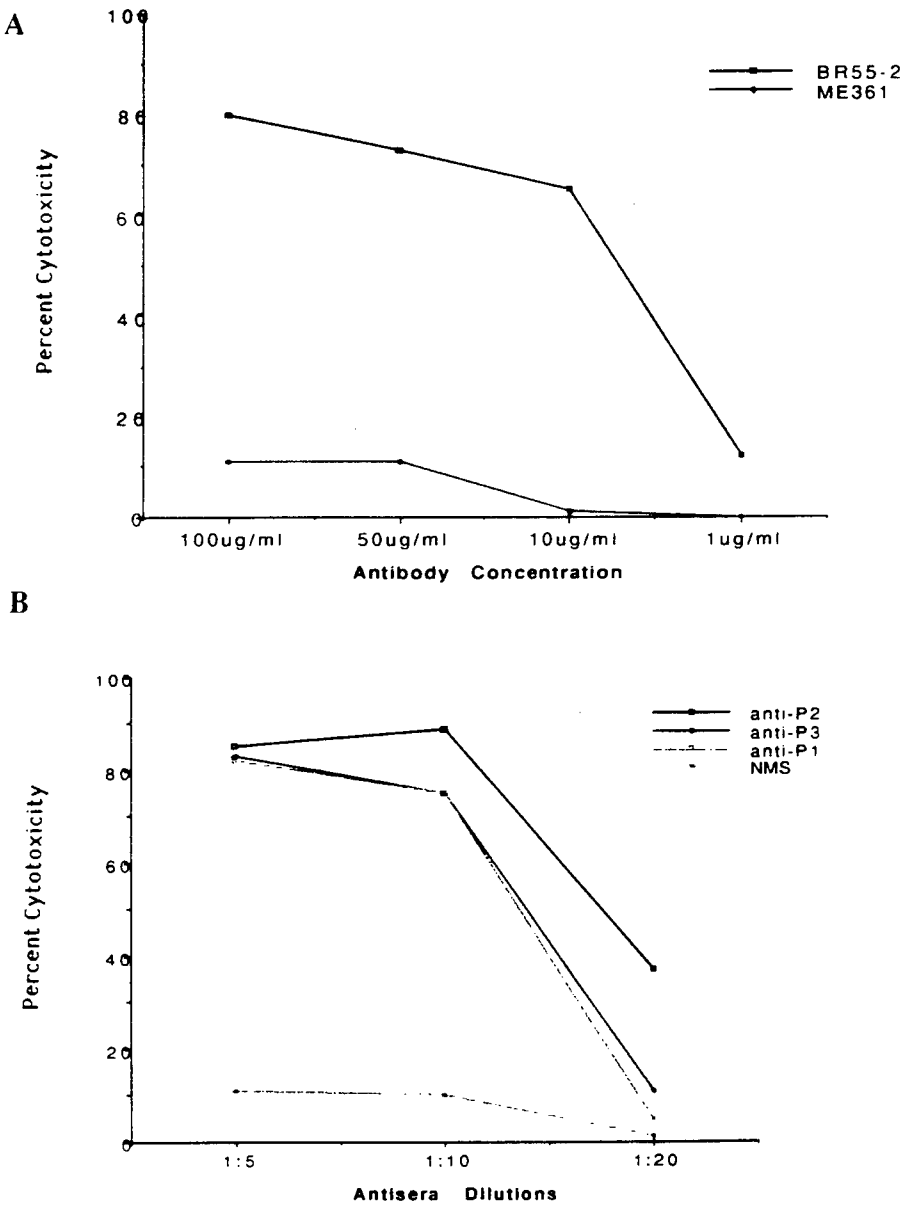

FIG. 4. CDC-mediated killing SKBR3 cell line. A. CDC killing by BR55-2. B. CDC killing by anti-peptide sera.

tered glycosylation, new epitopes appear on the cell surface that are absent in normal tissues.[37] Several investigators have shown that the expression of the STn epitope on mucins is associated with a poor prognosis in several human cancers, suggesting that STn may have functional significance in metastasis. Immunization with STn formulations has resulted in the observation that immunized patients can mediate complement-dependent cytotoxicity that parallels that observed with murine monoclonal antibodies.[5,24,25] This suggests that antibodies recognize the cancer-associated disaccharide NANA alpha (2 → 6)GalNAc. Evidence of a clinical response was noted in immunized breast cancer patients, with other patients showing prolonged disease stability.[5,24,25] By the same token, the difucosylated neolactoseries structure LeY, associated with several human tumors such as breast, lung, and gastrointestinal carcinomas[1–4,9–11,38] is also specifically detected by MAbs. These MAbs have unique properties among MAbs specific for solid tumors, *i.e.*, they are rapidly internalized and can effectively utilize human effector mechanisms (both cellular and complement-dependent) for tumor cell destruction and inhibit tumor growth in nude mice xenografted with human tumor cells.[34,49,40] However, there is no evidence that LeY is immunogenic in humans.

Because anti-carbohydrate antibodies reactive with carbohydrates associated with solid tumors (Fig. 1) mediate complement dependent cytotoxicity, we wanted to examine whether peptides that mimic carbohydrate forms can induce antibodies that are reactive with breast tumor-associated carbohydrates and whether this antisera can mediate breast tumor killing *in vitro*. Mice were immunized with peptide proteosome complexes and sera was found to be reactive with representative breast-associated carbohydrates (Fig. 2). It was further observed that the sera bound to SKBR3 cells in FACS analysis, with diminished reactivity with normal breast cells (Table 2). This activity is inhibitable by select synthetic probes (Fig. 3). We also found that the sera mediate complement-dependent cytotoxicity of the SKBR3 human adenocarcinoma cell lines at about 1:15 dilution (Fig. 4). The SKBR3 line expresses high levels of the LeY antigen, which can be targeted by both anti-LeY MAbs (Fig. 4A) and by the peptide antisera (Fig. 4B). On the basis of our present analysis, the YYPY motif appears to mimic better the LeY antigen subunits than the other motifs, because antisera to this motif are close to 40% inhibitable at 0.5 μg of synthetic probe (Fig. 3).

Very few groups are investigating carbohydrate-based vaccines or carbohydrate-based immunotherapy. One major reason for this is that carbohydrate antigens are expensive and very difficult to synthesize. Furthermore, expression of tumor-associated carbohydrate antigens is by no means specific to tumors. Crucial issues are expression of antigen density, multivalency, reactivity threshold of antibody binding, and efficient production of antibody having a strong complement-dependent or T-cell-dependent cytotoxic effect on tumor cells without damage to normal tissues. Studies on cancer vaccine development depend on many factors for success that include: (i) selection of carbohydrate epitopes; (ii) design and assembly of epitopes coupled to macromolecular complex as an efficient immunogen; (iii) establishment or availability of a good animal model; (iv) evaluation of immune response in animals; (v) tumor rejection without damage to normal tissues; and (vi) careful clinical application.

Because carbohydrate antigens are generally weakly immunogenic in humans, only short-lived IgM responses have been observed historically. The importance of adjuvant sublimation is highlighted in such studies to offset the relatively weak immunogenicity of carbohydrate structures.[6,15,17–21,23–25] In addition, antibodies to carbohydrates are typically of low affinity, and the notion of how cellular immunity is modulated by carbohydrates antigens is unclear. Subsequently, protein antigens have been viewed as more viable for vaccine development, and genetic vaccination is the most recent trend in such studies. The notion of using peptide mimics of carbohydrates to induce anti-carbohydrate immune responses parallels the use of anti-idiotypic antibodies as immunogens. Peptides that mimic carbohydrates might be used to augment naturally available immunoglobulins to tumor antigens. It is now also clear that humans with cancer have, in their draining lymph nodes, precursors of cytotoxic T cells that can be stimulated *in vitro* to react against their tumors.[37] Peptide formulations might trigger such precursors. Subsequently peptides that mimic tumor-associated carbohydrates would be of importance as novel agents for adjuvant therapy.

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Peptide mimicry of carbohydrate epitopes on human immunodeficiency virus

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Cancer-related, mucin-type carbohydrate epitopes, principally mannose and sialo-syl residues, are expressed on the envelope protein gp160 of the human immunodeficiency virus (HIV). Anticarbohydrate antibodies directed toward these and other carbohydrate epitopes are known to neutralize HIV-1 infection by cell-free virus. Carbohydrates, however, being T cell–independent antigens, typically elicit diminished immune responses. To overcome this potential draw back, we have examined the ability of peptides that mimic such epitopes to elicit immune responses that cross-react with carbohydrate structures. We report that mouse polyclonal antisera generated against peptides that mimic mucin-related carbohydrate epitopes have anti–HIV-1 activity. Generation of antibodies was not Ir-gene restricted, as at least two different strains of mice, Balb/c (H-2^d) and C57Bl/6 (H-2^b), responded equally to the peptides. The antipeptide sera displayed neutralizing activity against HIV-I/MN and HIV-I/3B viral strains. This neutralization was as good as human anti-HIV sera. These results indicate that peptide mimics of carbohydrates provide a novel strategy for the further development of reagents that elicit immune responses to carbohydrate epitopes associated with many infectious organisms and tumor cells.

Keywords: applied immunology, peptide mimetic, carbohydrate, polysaccharide, HIV-1, Lewis Y

Protein-carbohydrate interactions mediate the initial steps in many bacterial and viral infections. The envelope (env) glycoprotein of human immunodeficiency viruses (HIV-1 and HIV-2) interacts with target cells through high mannose and/or N- and O-glycosylated regions of gp160 (ref. 1). Subsequently, certain lectins and anticarbohydrate antibodies display the capacity to neutralize HIV field and laboratory isolates in vitro[2–6]. O- and N-linked carbohydrates are one set of common saccharide subunits shared among bacteria, viruses, and tumor cells. Antibodies that target the Tn (GalNAc-Ser/Thr), sialo-syl-Tn (NeuAc-GalNAc-Ser/Thr), and the Histo-blood group antigen Lewis Y, (Fucα1→2 Galβ1→4(Fucα1→3) GlcNAcβ1→3Galβ1→4Glcβ1→R), occurring as surface antigens on most primary human breast carcinomas and their metastases, inhibit HIV infection and syncytium formation[5]. These observed cross-reactivities for HIV and tumor cell-associated carbohydrates suggest that the pathophysiology of infection and neoplasia are profoundly affected by the same or similar carbohydrate forms.

Viral-borne carbohydrates that are not structurally encoded by the viral genome represent a target for group-specific vaccine development as these antigens are unlikely to change dramatically with viral mutation. Unfortunately, carbohydrates are, per se, not immunogenic in humans and require extrinsic adjuvanticity, as they suffer from an inherent inability to generate antigen-specific T cell responses. Immune responses can be enhanced by coupling carbohydrates to immunologic-carrier proteins or administering them with adjuvants[7]; however, synthetic antigen-conjugates (representative of those on the HIV-1 env) do not always induce immune responses reactive with native antigens[8,9].

To overcome this and related deficiencies, surrogate peptide antigens might prove effective for eliciting immune responses reactive with natural carbohydrate forms[10]. In this context peptide mimetics have a significant conceptual advantage for vaccine design. As peptides, they have the ability to stimulate T cell help in an antigen specific manner. Ultimately, such a vaccine should be able to generate long-term immune responses, and would have advantages for manufacturing and vaccine production. The ability of a peptide or polypeptide to immunologically mimic a carbohydrate determinant[11–15] indicates that while mimicry is accomplished using amino acids in place of sugars, the specificity pattern can be precisely reproduced.

We show that immunization with peptides that mimic mannose and lactoseries carbohydrate subunits induce antibodies that cross-react with native HIV env proteins and can neutralize HIV-1 infection. These studies substantiate that induced antibodies to common carbohydrate subunits found on bacteria and tumor cells can also bind viral glycoprotein(s). Peptide antigens that are mimics of carbohydrate antigens thus provide an alternative vaccine strategy to elicit an appropriate immune response against natural polysaccharides.

Results

Choice of peptides. Peptides were chosen based upon the distribution of mucin-type and peripheral poly-N-acetylglucosamine carbohydrates on the major env protein of HIV-1 (refs. 4, 5, 16). Peptide motifs identified to mimic these carbohydrate forms are

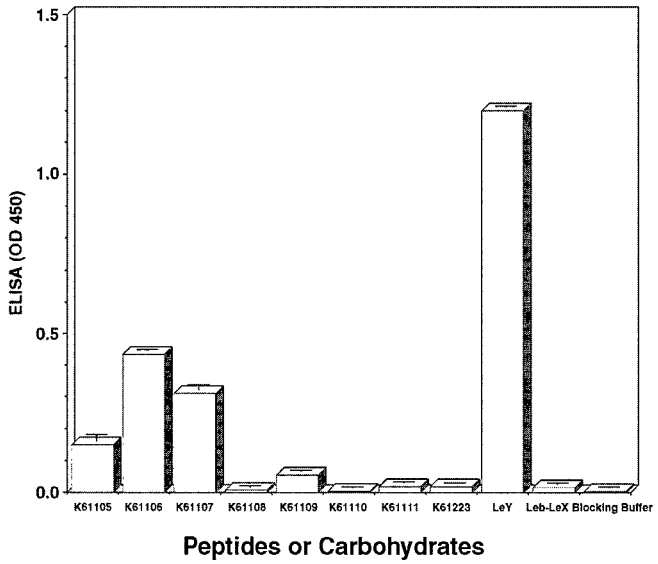

Figure 1. Binding of the LeY specific antibody BR55-2 to various peptides and LeY. The peptides K61105, K61106, and K61107 correspond to YYPYD, YYRYD, and YWRYD respectively. Other K series peptides were variants of the motifs or irrelevant peptides. Blocking buffer alone was also used as a control because anticarbohydrate antibodies have a tendency to adsorb to blocking agents, enhancing nonspecific binding.

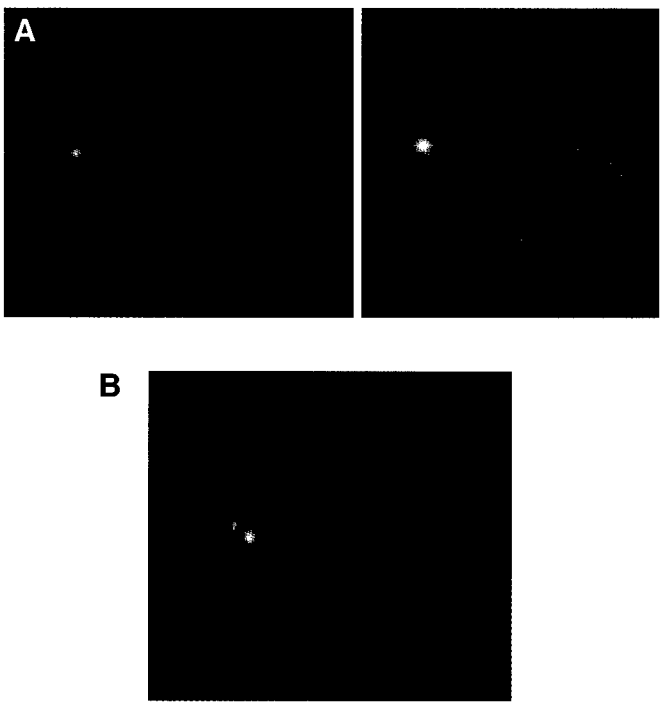

Figure. 2 Functional group similarities between LeY and MCP. (A) Low-energy conformers of LeY (on left side of panel A) and MCP (on right side of panel A) are compared highlighting the conserved spatial positions of the methyl group on GlcNAc (magenta colored sphere) and hydroxyl oxygens on the Fuc residue of Fuc1-3GlcNAc (red colored spheres) of the LeY structure and the methyl group of α2 sialic residue (magenta colored sphere) and hydroxyl oxygens (red colored spheres) of α9 MCP. (B) Superposition of LeY tetrasaccharide and MCP. In this orientation, the hydroxyl groups on the Fuc residue of Fuc1-3GlcNAc are spatially conserved with those of the α9 sialic residue of MCP, while the respective methyl groups on GlcNAc and on α2 sialic residue are spatially conserved.

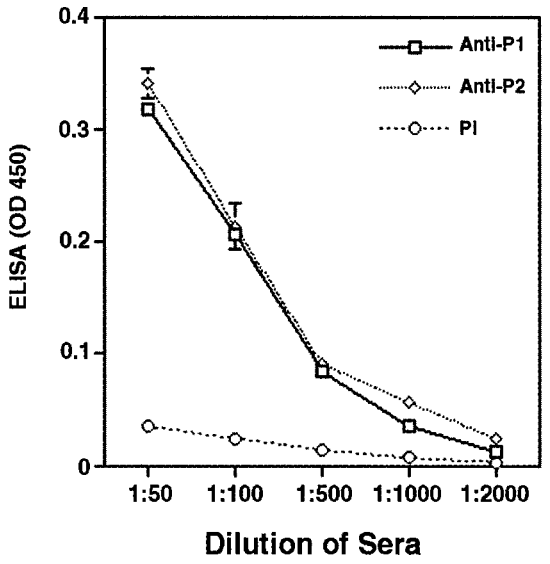

Figure 3 Binding of polyclonal antisera to LeY.

YPY, which has been found to mimic mannose[17,18]; WRY, which has been found to mimic α(1-4)glucose[19,20]; PWLY, found to mimic LeY[21]; and YRY, found to mimic the major C polysaccharide α(2-9) sialic acid (MCP) of *Neisseria meningitidis*[10]. It is noteworthy that these potential surrogate immunogens involve aromatic-aromatic interactions, suggesting that the motifs mimic carbohydrate subunits shared among a variety of carbohydrate forms.

The antigenic mimicry of LeY by the planar-X-planar motifs is shown in Figure 1. ELISA reactivities of YPY, YRY, and WRY motifs with the anti-LeY monoclonal antibody BR55-2 indicate that BR55-2 is specific for these motifs, displaying very little reactivity with other peptide sequences. BR55-2 displays high specificity for LeY, being made against an LeY-expressing tumor cell line[22]. We have recently determined the molecular recognition properties of LeY for BR55-2 (ref. 23), and that the LeY tetrasaccharide core structure is similar to the core structure of MCP (Fig. 2). The low energy-conformations of MCP and LeY structures overlap in their antigenic presentation, which may be mimicked by homologous peptides (Fig. 1).

Anti-LeY response to peptides. To determine the extent to which aromatic-aromatic motifs immunologically mimic the LeY antigen, we immunized Balb/c and C57Bl/6 mice with peptides containing YYPYD (P1) and YYRYD (P2) motifs. We also immunized with a peptide that changes the YYRYD sequence tract to YYRGD (P3). The RYD sequence has been shown to be a mimic for the adhesion motif RGD and its conformational

Table 1. Mean florescence of binding of antipeptide sera to different cells as measured by FACS.

Cell line	Anti-P1 (YYPY)	Anti-P2 (YYRYD)	Anti-P3 (YYRGD)
SKBR3	240.6	275.6	166.7
HS578 Bst (normal breast)	17.8	19.9	22.4
WM793	145.5	172.4	42.3
MT2	19.5	22.3	23.1

Background fluorescence (mean fluorescence) associated with nonspecific mouse sera is 24.2 for SKBR3, 24.4 for WM793, 17.3 for MT2, and 18.4 for HS578 (final sera concentration: 1:50).

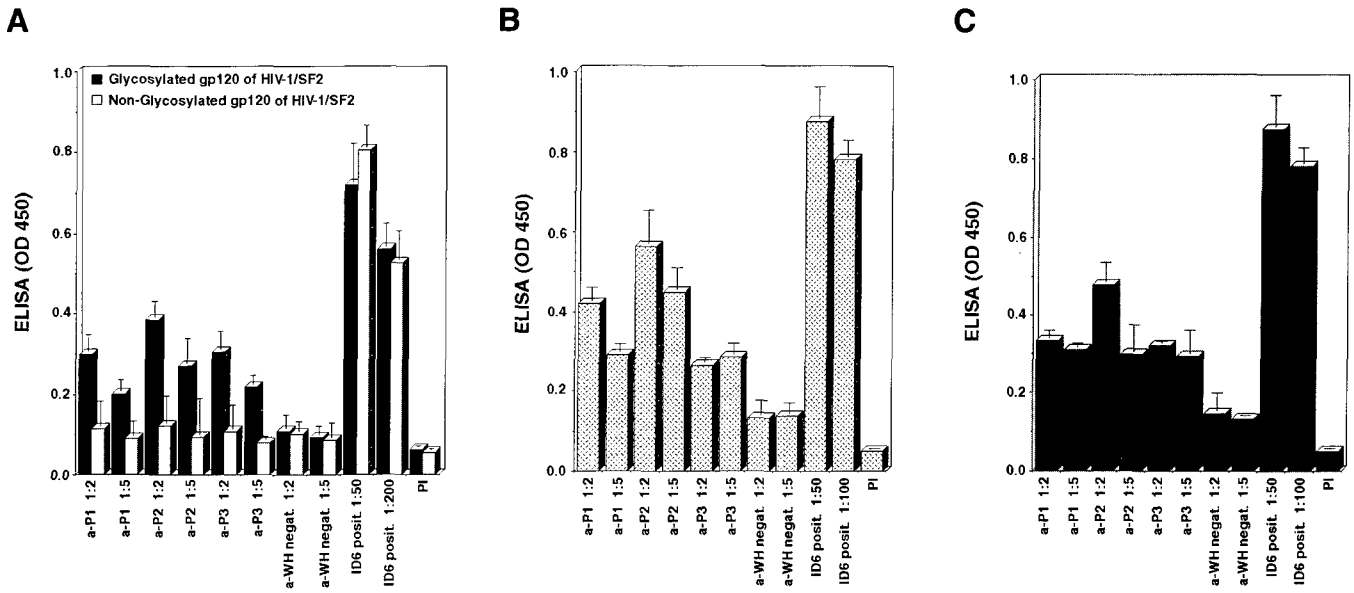

Figure 4. Binding of polyclonal antipeptide sera to HIV-1/SF2 and MN env protein. (A and B) Binding of sera derived from C57Bl/6 immunized mice; (C) Binding of sera derived from Balb/c mice. PI in (A and B) is preimmune C57Bl/6 sera. PI in (C) is preimmune Balb/c sera.

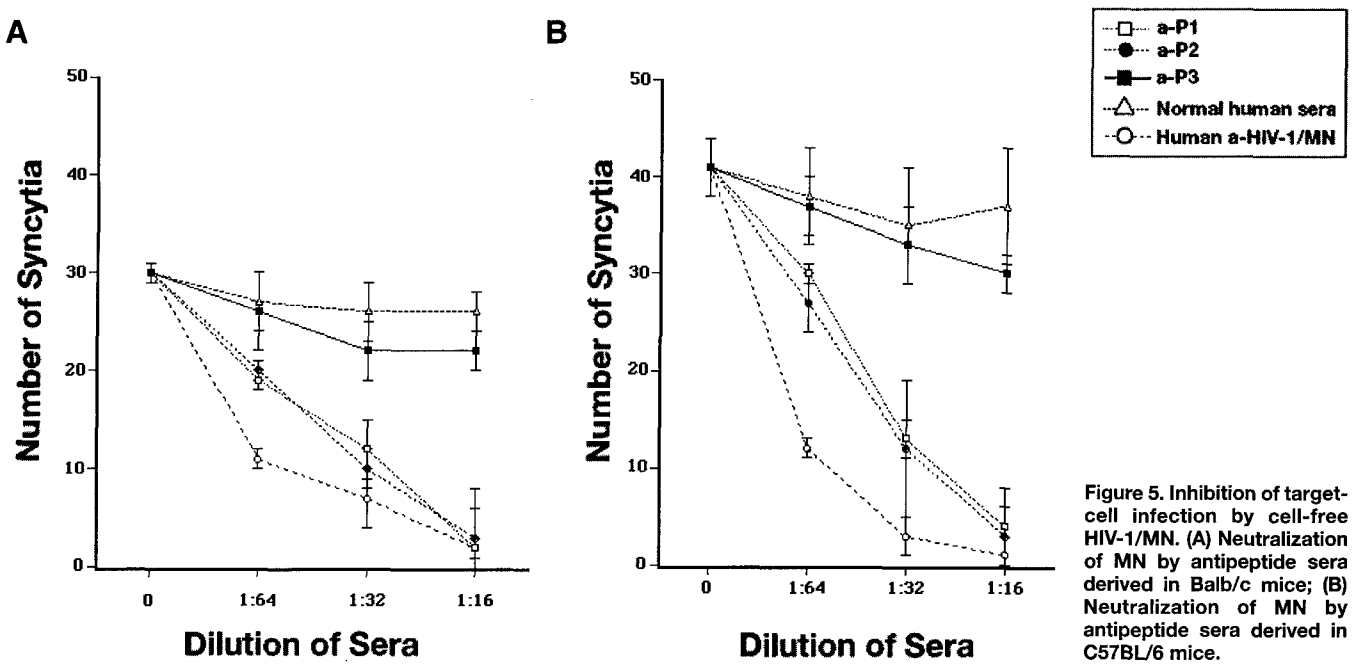

Figure 5. Inhibition of target-cell infection by cell-free HIV-1/MN. (A) Neutralization of MN by antipeptide sera derived in Balb/c mice; (B) Neutralization of MN by antipeptide sera derived in C57BL/6 mice.

properties correlate with bioactive RGD compounds[24]. Peptides had the form CARIYYXYDGFAY, which followed the structural properties of an antibody loop[10] or were synthesized as triple repeating units that emulate helical configurations, often observed for polysaccharides. It was observed that the reactivity of the IgG antisera to P1 and P2 titers out to 1:2000 against the LeY structure on solid phase ELISA (Fig. 3) with little reactivity observed from preimmune (PI) sera.

Envelope protein binding. We examined whether the polyclonal antipeptide sera derived from C57Bl/6 bound to glycosylated and nonglycosylated forms of HIV-1/SF2 (Fig. 4A). Negative-control groups included preimmune sera, a mouse polyclonal monospecific antisera directed to an irrelevant antigen[25] and a positive-control Balb/c monoclonal antibody (ID6) directed to HIV-1 glycosylated and nonglycosylated gp120 (ref. 26). Unexpectedly, we found that reactivity with the glycoslyated forms of HIV-1 env provided statistically significant O.D. readings only up to 1:5 dilution. Nevertheless, within this dilution range we observed that the sera bound approximately threefold better to the glycosylated form of SF2 over preimmune sera background, while binding to the nonglycosylated form was equivalent to that observed for the negative-control murine sera derived from immunization with an irrelevant antigen.

We also examined this sera for binding to glycosylated gp140 env protein of HIV-1/MN, lacking the transmembrane domain of gp41 (Fig. 4B). The antisera against all three peptide motifs bound

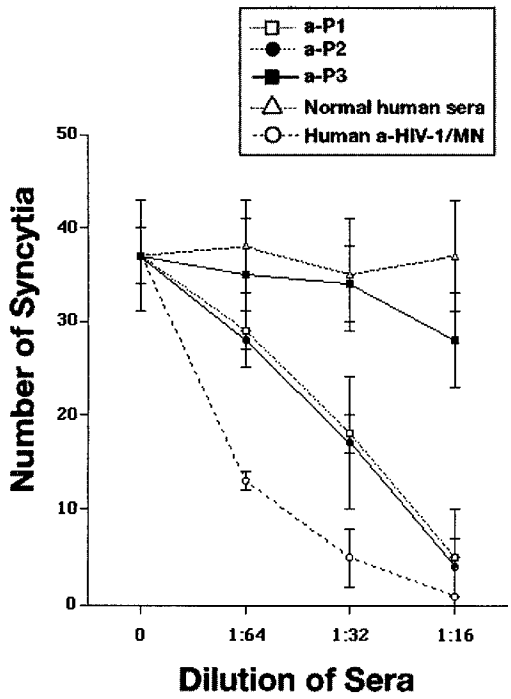

Figure 6. Inhibition of target cell infection by cell free HIV-1/3B isolate with sera derived from Balb/c mice.

to gp 140 HIV-I/MN when used at final dilution of 1:5. Binding was at least twofold above the preimmune sera and sera derived from immunization with irrelevant antigen as background. The same result was observed with sera derived from Balb/c animals (Fig. 4C). These results suggest that the antisera could recognize sugars on the HIV env protein, but perhaps the sugars were not presented in a proper orientation for maximum antisera reactivity to occur, or that the density of the carbohydrate expressed on the env protein surface was not high. In any event, our antisera bound to glycosylated but not to nonglycosylated gp120 derived from HIV-1 SF and MN.

Neutralization of HIV. Sera from Balb/c (Fig. 5A) and C57Bl/6 (Fig. 5B) mice immunized with P1 or P2, neutralized HIV-1/MN at final dilution's up to 1:64. Normal human sera and the anti-P3 sera were ineffective at blocking syncytia formation, whereas human α-HIV-1 sera from four different infected patients neutralize cell-free virus at dilutions up to 1:64. The isolate specificity was further determined by cell-free neutralization of the HIV-1/3B isolate (Fig. 6). As with MN, anti-P1 and anti-P2 Balb/c sera were effective in neutralizing virus infection in vitro. Collectively, these results suggest that the production of HIV-neutralizing antibodies by the peptide-proteosome complexes induces humoral immune responses in divergent haplotypes that can be as effective as sera from HIV-1–infected individuals in neutralizing HIV-1 cell-free infection.

Binding of immune sera to cells. To further confirm that the peptide-induced antisera were reacting with virus but not target cells used in the neutralization assay, we investigated binding of the sera to different cell lines including the MT-2 human T cell line by FACS. All three antisera did not bind to the MT-2 line at all (Table 1). Importantly, the antisera also did not bind to normal breast cells, HS578, which suggests that there is little to no tissue adsorption of the antipeptide sera in normal tissue. All three sera reacted very strongly with the LeY-expressing SKBR3 human breast-cancer line. Two out of three antisera also bound the human melanoma cell line WM793, which expresses sialyated GD2/GD3 gangliosides. While the antisera recognized overly expressed carbohydrate antigens on two control cell lines, no binding to the target MT-2 cells was observed, suggesting that the mechanism of inhibition of viral infection is connected with binding of the antipeptide sera to viral particles.

Discussion

Comparison of oligosaccharide profiles on the HIV env protein reveals that different virus isolates, propagated in the same host cells, yield very similar glycan patterns, whereas cultivation of an isolate in different host cells results in markedly divergent oligosaccharide maps[27]. Variations concern the proportion of high-mannose-type, hybrid-type, and complex-type substituents, as well as the state of charge and structural parameters of the complex-type species. As a characteristic feature, complex-type glycans of monocyte-derived–macrophage-derived viral glycoprotein are almost exclusively substituted by lactosamine repeats.

We and others have found that certain peptides mimic carbohydrate subunits, inducing cross-reactive in vivo anticarbohydrate-like antibody responses. We have shown that aromatic containing peptide motifs can mimic salient features of at least one lactosamine form, LeY. Unlike synthetic carbohydrate forms[8,9], sera induced with these peptide motifs bound to an LeY-expressing cell line and can neutralize HIV-1 cell-free infection in vitro. The specificity in the neutralization profile is illustrated by considering that a change in one amino acid in an immunizing peptide (YYRYD to YYRGD) can affect the neutralizing ability of the antipeptide sera. We have found that the production of HIV-neutralizing antibodies was not severely major histocompability complex-restricted, and cross-reacts and cross-neutralizes with at least two divergent isolates within clade B; the major North American and Western European clade. We do not know at this time if the antipeptide sera inhibit primary isolates of HIV-1, which are more resistant to neutralization[27–30].

It was not the intent of these studies to advocate a new vaccine that displays broad HIV-1 neutralization ability, but rather to draw attention to the possibility of developing peptides that mimic HIV-1–associated carbohydrate forms. We found that aromatic-aromatic interactions are a major driving force in mimicking carbohydrate subunits. Polymerization of aromatic residue-containing peptides can structurally mimic the helical shape of many carbohydrate forms. While carbohydrate-conjugate vaccines are certainly effective and are viewed as superior to peptide mimics, peptide mimics might be used as priming or boosting agents, being formulated to develop longer-lasting responses after booster immunization. As peptides have the ability to stimulate T cell help in an antigen-specific manner, peptide mimetics would be of importance as novel agents for adjuvant therapy. Peptides that mimic carbohydrates may be further designed and manipulated to develop specific immune responses against a variety of polysaccharides on bacteria, viruses, and tumors that might be associated with their pathobiology.

Experimental protocol

Generation of polyclonal sera. Peptides were synthesized with the addition of a tripeptide YGG spacer, and a cysteine at the amino terminus conjugated to a lauroyl group (Bio-Synthesis, Lewisville, TX) and then complexed with proteosomes[10]. For generation of polyclonal sera, Balb/c mice (H-2^d) (four per group) and C57Bl/6 (H-2^b) (four per group) 4 to 6 weeks of age, were immunized intraperitoneally on a weekly basis for 3 weeks with 50 μg of peptide-proteosome complex. Sera were collected within 7 to 14 days after the last immunization and analyzed for binding against LeY by ELISA .

Binding of immune sera and BR55-2 to LeY and peptides. Solid-phase ELISA was performed to asses the binding activity of the generated sera to LeY incorporated into polyacrylamide (PAA) matrix, creating 30 kDa multivalent polymer (GlycoTech Inc., Rockville, MD). Immunlon 2 plates were

coated with the multivalent LeY-PAA probe overnight at 4°C. The plates were blocked with 0.5% FCS/0.2% tween , 200 µl/well, 37°C, for 1 h. Serial dilutions of the respective antisera were added and incubated at 37°C for 2 h and resolved with 100 µl/well of 1:10,000x goat antimouse IgG isotype matched HRP (Sigma, St. Louis, MO) diluted in blocking buffer, incubated at 37°C for 1 h, and read at OD_{450}. This same protocol was used in assessing BR55-2 binding to various peptides and carbohydrate probes. Peptides were presented as MAP peptides (Genetics Research, Huntsville, AL) coated at 2 µg/well. Multivalent LeY and Leb-LeX probes were coated at 0.1 µg/well and the monoclonal antibody BR55-2 (IgG3) concentration used was 0.1 µg.

Binding of immune sera to cells. Cells, with FACS buffer (1% BSA, 0.01% Na azide, 25 mM EDTA) were washed, scraped and transferred to 15-ml centrifuge tubes. Viability of cells was checked by trypan blue and 100 µl of 1 to 2×10^6/ml cells in FACs buffer were used for analysis. Ten microliters of experimental or control sera were added to sample tubes and incubated on ice for 30 min, washed twice and 10 µl of FITC Ab (goat antimouse IgG conjugate FITC-labeled (Sigma) diluted 1:20 with PBS) was added to the sample. Cells were fixed with 1% paraformaldelhyde, followed by FACS measurement.

Binding of immune sera to HIV envelope protein. Oligomeric soluble gp140 , a truncated env of the HIV-1/MN, was produced by BS-C-1 cells infected with VPE12B, recombinant vaccinia virus, and was purified[31]. Bacteria produced nonglycosylated SF2 gp120 and Chinese hamster ovary cells produced glycosylated SF2 gp120 were obtained from the AIDS Research and Reference Reagent Program (Rockville, MD). Binding of sera were determined by ELISA as previously described[26]. Briefly, 2 µg/ml of recombinant proteins were adsorbed onto microtiter wells. Serial dilutions of experimental or control antisera were added to these antigen-coated plates. Wells were washed, incubated with goat antimouse IgG conjugated with HRPO (Sigma), and developed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB).

Viral neutralization assay. Cell-free HIV-1/MN and HIV-I/3B, obtained from the AIDS Research and Reference Reagent Program, were propagated in H9 cells. Cell-free virus neutralization was performed as previously described[32,33] with minor modifications. One hundred $TCID_{50}$ of HIV-1/MN or HIV-1/3B cell-free virus (50 µl) were preincubated with serial dilutions of experimental antisera or controls (preimmune mouse sera, normal human sera, or mixture of four sera of HIV-1 positive patients) for 1 h at 37°C. Following incubation, the pretreated virus was then plated on 4×10^4 HTLV-I/MT-2 target cells (50 µl), for 1 h at 37°C . The target cells were then washed three times and incubated at 37°C with 5% CO_2. Neutralization was detected as inhibition of syncytia 3 days later, assessed by counting the number of multinuclear cells[33].

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Specificity Analysis of Polyclonal Sera Rasied to Peptide Mimics of Tumor Associated Carbohydrate Antigens

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Abstract

Recently, we found that peptides containing aromatic residues can mimic mucin or histo-blood group related carbohydrate epitopes, eliciting polyclonal responses reactive against bacterial or viral antigens that express such carbohydrate forms. These peptide forms include the sequences GGIYYPYDIYYPYDIYYPYD, GGIYWRYDIYWRYDIYWRYD, and GGYYRYDIYYRYDIYYRYD. Our data demonstrates that peptides can function as carbohydrate surrogate antigens. To further study the nature and biologic significance of the antigenic and immunological mimicry between carbohydrate and peptide antigens, these peptides were reacted with monoclonal antibodies (MAb) that recognize biologically important conformations of the Lewis Y antigen expressed on the surface of adenocarcinoma cells. Results by ELISA demonstrate that the MAbs can distinguish particular peptide motifs. Binding of MAb to Lewis Y is peptide inhibitable. We also generated polyclonal antibodies against these peptides using either peptide-proteosome conjugates or as Multiple Antigen Peptides (MAPS) administered with QS-21. Sera was observed to react with synthetic carbohydrate subunit probes representative of mucin and histo-blood group related antigens. Immunologic presentation of the peptides as MAPs improves upon their ability to induce Lewis Y specific immune responses, particularly for the YYRYD sequence tract. Sera was found to bind only to tumor cells that expressed carbohydrate and mediated tumor cell killing by complement mediated cytotoxicity. Results of our experiments indicate that peptides can both antigenically and immunologically mimic tumor associated carbohydrate antigens, inducing anti-tumor immune responses.

Introduction

Carbohydrates play an essential role in cell biology being involved in cell-cell communication, cell proliferation and differentiation (cell growth). The aberrant expression of branched and sialylated complex-type N and O-linked oligosaccharides in malignant tumor cells appears to be directly associated with metastatic potential (1, 2). The expression of both simple and complex carbohydrates predicts unfavorable prognosis in breast and other cancers (3, 4). The blood group-related neolactoseries carbohydrate structures Lewis X (LeX), sialyl-LeX (sLeX), Lewis a (Lea), sialyl-Lea (sLea) and Lewis Y (LeY) are examples of terminal carbohydrate structures related to tumor prognosis (4, 5). These antigens constitute carbohydrate moieties of tumor associated gangliosides, the human carcinoembryonic antigen family, the human pancreatic MUC-1 antigen and are identified in carcinomas of the skin, stomach, pancreas, lung, colon, breast and prostate. The histo-blood group related antigens sLeX and sLea, are also implicated as immunogenic antigens in human melanoma (6). The T (Thomsen Friedenreich (TF)) Galβ1$\rightarrow$3GalNAcα, Tn precursor GalNAcα, and its sialylated form (STn) are powerful histologic markers in diagnosis and prognosis, occurring as surface antigens on most primary human breast carcinomas and their metastases (7-11) These observations indicate that carbohydrates play an important role in cancer biology and are prominent targets for immunotherapeutic strategies for cancer treatment.

Tumors may actually express a complex profile of related but distinct glycans sharing similar terminal immunodominant sugar residues, such as GalNAc on breast cancers, which may be implicated in aggressive biological behavior (11). Conformational analysis of tetrasaccharide moieties suggest that reactivity of a common carbohydrate epitope with different antibodies or ligands is highly dependent on the type of carrier glycosylceramide or carrier O-linked peptide (12). These latter results suggest that antigenic expression (physical conformation and orientation) on the cell surface is governed by the nature of the carrier glycosylceramide or carrier O-linked peptide. Therefore, antibodies induced by synthetic carbohydrate formulations might not be reactive with natural antigens on a tumor surface (10, 13, 14). This is a major concern for vaccine design using synthetic carbohydrate antigens (15).

Most carbohydrate antigens belong to the category of T cell independent antigens that reflect their inability to stimulate MHC class II dependent T cell help (16). Consequently, carbohydrates are not capable

of induction of a sufficient anamnestic or secondary immune response. Furthermore, antibodies produced in response to carbohydrate antigens usually are not of high affinity compared to those produced by responses to peptide or protein antigens. An alternative approach for augmentation of carbohydrate immunity is the use of anti-idiotypic antibodies (17, 18) or peptide surrogate antigens (19, 20). This approach requires that the antigenic mimicry accomplished using amino acids in place of sugars, induces a specificity pattern for the nominal carbohydrate antigen that can be precisely reproduced (17, 18, 21).

We have shown that peptides can function as surrogate immunogens for carbohydrates expressed on bacteria (22), on the envelope protein of HIV-1 (23) and on adenocarcinomas (24). Here, we further delineate specificity patterns associated with the antigenic and immunological mimicry of peptide mimotopes of LeY. Contrasting the antigenic and immunological properties of peptide sequences that mimic carbohydrate forms provides information about how amino acid differences lend to specific antigenic and immunological mimicry. We observe that the presentation of peptide mimotopes influences the specificity pattern for histo-blood group synthetic carbohydrate probes associated with human tumors. We also observe that peptide mimotopes elicit polyclonal sera that specifically bind to human tumor cells but not to normal tissues, and can mediate complement dependent cytotoxicity (CDC) of representative human breast cell lines, albeit at low titers.

Results

Antigenic mimicry of peptide motifs

Peptides containing putative aromatic-aromatic motifs have been defined to mimic several carbohydrate subunits (Table 1). These peptides demonstrate the preference of aromatic groups separated by an intervening residue. Peptide motifs identified to mimic these carbohydrate forms are YYPY as a mimic of mannose as identified from peptide phage screening with Con A (25, 26), WRY found to mimic α(1-4)glucose as identified from analysis of protein that bind to α-amylase [Murai, 1985 #606;Mirkov, 1995 #605], PWLY found to mimic LeY as identified from peptide phage screening with an anti-LeY antibody, B3,(27), and YYRYD derived from an anti-idiotypic antibody found to mimic the major C polysaccharide α(2-9) sialic acid (MCP) of *Neisseria meningitidis* (22).

The sequence similarities among the putative motifs suggest that antibodies raised to this peptide set might cross-react with similar subunits expressed on what are otherwise dissimilar carbohydrate structures. For example, polyclonal antibodies raised against the motif YYRYD might cross-react with MCP and with LeY. Molecular modeling suggests that the LeY tetrasaccharide structure is similar to the core structure of MCP, providing a structural basis for potential cross-reactivity (23). To further determine the extent of cross-reactivity for these motifs peptides were synthesized repeating the respective putative centralized motifs shown in Table 1. It is theorized that the repeating tract should adopt a helix configuration which emulates many extended carbohydrate structures. To evaluate the antigenic mimicry of motif forms, we synthesized respective MAP forms for detection of a reactivity patterns with the anti-LeY monoclonal antibodies BR55-2, and 15.6 and the anti-ganglioside antibody ME361 (Figure 1a). Significant reactive sequences in Figure 1a correspond to 3 peptides, GGIYYPYDIYYPYDIYYPYD (k61105), GGIYWRYDIYWRYDIYWRYD (K1106) and GGYYRYDIYYRYDIYYRYD (K61107). Other peptides include a triple repeat of the APWLY motif reactive with another anti-LeY antibody B3 (27) - e.g. GGGAPWLYGAPWLYGAPWLY (K61223) and a derivatized form were not reactive with these antibodies.

BR55-2 bound very well to K61106 and K61107 relative to the other peptides. Unlike 15.6A, the monoclonal ME361 also reacted with these peptide forms, displaying O.D.s for K61105 and K61110 similar to that observed for the synthetic LeY antigen. The reduction in reactivity of 15.6A for peptides otherwise reactive with BR55-2 suggests that the peptides mimic a structural feature(s) unique to BR55-2 recognition. BR55-2 and 15.6A show distinct binding properties for LeY expressing tumor cells. In addition, BR55-2 displays little reactivity with the K61223 and K61108 peptides which represents the APWLY motif reactive with the ant-LeY antibody B3 (27). The affect of sequence on reactivity is observed with lack of reactivity of BR55-2 with K61109 in which the WRY tract was synthesized in a different molecular environment. This data further suggests that K61106 and K61107 mimic salient features of the surface conformation of LeY which is compatible with the BR55-2 combining site since BR55-2 selectively cross-reacts with these peptides. Inhibition of LeY-PAA binding of BR55-2 by these MAP peptides is shown in Figure 1b. K61106 and K1107 displayed 50% inhibition of BR55-2 binding to LeY with 20

times molar excess. These data indicate that the YRY and WRY motifs synthesized as a triplet, lend to reactivity of these motifs with BR55-2. Substitution of YPY (K61105) reduces the recognition ability of BR55-2.

The induction of anti-carbohydrate immune responses by peptides.

The above mentioned possible structural similarities suggest that anti-sera raised to the peptide putative motifs might cross-react with a variety of subunits representative of Lewis antigens. The immunological presentation of the putative motifs, (i.e. short or longer peptides, presentation in a helix or beta bend) might mimic overlapping epitopes on otherwise different carbohydrate structures. To test this idea, Balb/c mice were immunized with peptide-proteosome conjugates representative of the motifs YYPYD (P1), and YYRYD (P2) or the same peptides as MAP forms administered with QS-21. Sera were collected 1 week after the last immunization, pooled and tested for reactivity with LeY and Leb. For the proteosome conjugates we found that sera developed from the immunizations react with the two multivalent probes, with the IgG reactivity titering up to 1:2000 (figure 2a). Superposition of LeY and Leb structures indicate that despite the change of glycosidic linkage from β1-3 to β1-4 in the type 1 and 2 chains, resulting conformational features of the respective sugar moieties are still shared forming a common topography (28). The only effective difference is the position of the N-acetyl and hydroxymethyl groups projected on opposite sides of the type 1 and 2 difucosylated structures. The ELISA results in figure 2a suggest that the sera is reacting with the common topography of LeY and Leb.

Sera derived from immunization with the MAP peptides indicate that the IgM isotype is the predominate form, titering out to 1:2000 for the two carbohydrate forms, but displaying significant differences in reactivity for LeY and Leb that are accentuated at 1:100 and 1:500 titer (figure 2b). In contrast to the IgG pre-immune sera, the IgM fraction of the pre-immune sera consistently displayed higher reactivity with the carbohydrate probes, enhancing the non-specific binding (data not shown).

To further evaluate the specificity of the anti-carbohydrate IgG fraction derived from proteosome-conjugate immunized mice, ELISA assays were performed with plates coated with various synthetic carbohydrate subunit probes (Figure 3a). For BR55-2, selective binding was observed for LeY. ME361 was not reactive with any of the synthetic probes. It was observed that the anti-peptide sera (1:100 titer) was

reactive with respective carbohydrate probes above background binding. Preferences for antisera against the YPY motif include Fucα1-3GlcNAc representative of LeY, and Lex, the H type 1 structure Fucα1-2Galβ1-3GlcNAc representative of Leb, and the disaccharide Galβ1-4Glc. For the YRY motif, reactivity again was observed for all of the synthetic probes, with the disaccharide Galβ1-4Glc displaying the highest reactivity. The antisera reacted with both H type 1 and H type 2 (Fuca1-2Galb1-4GlcNAc) structures, with the type 1 structure displaying slightly more activity. This may reflect interaction with the common Fucα1-2Gal structure found on type 1 and type 2 structures or functional groups that are shared within the common topography of these two chains. Reactivity was observed for the TF representative subunit Galβ1→3GalNAc, and the Leb and Lea Fucα1→4GlcNAc subunit. These data suggest that there is a large degree of overlap in the potential carbohydrate structures being recognized by the antisera which was expected since the peptides mimic a wide range of singular carbohydrate subunits.

In contrast to the proteosome-peptide conjugates, increased specificity for LeY is observed for the MAP forms (Fig. 3b). At 1:50 dilution, the LeY reactivity of the antisera is approximately 3 fold more reactive with LeY than with Leb, Lex-pentasaccharide, SLea, SLex. Antisera to the YYRYD containing MAP (K61107) displayed about 1 fold less reactivity with Lea and Sialyl-Lex. Considering the diminished reactivity against LeX which shares the Galβ1→4(Fucα1→3)GlcNAcβ1 component it would appear that a fraction of antibodies react with a conformational component of LeY, providing for its increased reactivity, or alternatively, the affinity for LeY is increased. For either reason, these data suggest that multivalent or multiple antigen peptide forms might provide increased selectivity or avidity for polyvalent or clustered Lewis forms.

Distribution of sera reactivity.

An important consideration in the development of synthetic surrogate immunogens is the reactivity of the induced IgG sera to react with naturally expressed carbohydrate on the tumor surface. We have evaluated the ability of IgG elicited by peptide-proteosome, compared with IgM fractions induced by MAP forms of the same peptides, to bind to representative tumor cells as evaluated by FACS assay (Table 2). Positive control monoclonals were BR55-2 and ME361. Normal mouse sera (NMS) and sera generated against proteosome alone were also used as a controls. Of interest was whether induced predominant IgG or IgM

sera react the same. We found that sera from MAP peptide immunized mice displayed a higher mean fluorescence for MCF7 cells than the proteosome formulation. For SKBR3 cells both sera types reacted about the same. Both sera types displayed minimal reactivity with the normal breast cell line and murine fibroblast. Anti-YYRYD and Anti-YYPY sera reacted with WM793 cells but to different degrees. Melanoma cells have recently shown to express sLeX and sLea (6). We tested the WM793 cells by FACS with the antibody FH-6 which is specific for sLeX. Reactivity was observed for this antibody (data not shown). Our results suggest that the respective sera is perhaps cross-reacting with sLeX on the WM793 cells since we see some reactivity with this probe (Figure 3a).

We extended our examination for cross-reactivity to include both normal tissue and tumor specimens. Rabbit sera to the P2 peptide was chosen to screen normal tissues because this peptide elicits sera which displays reactivity for MCP and LeY. Therefore it may be perceived that this peptide might induce sera with broad reactivity for a variety of carbohydrates expressed on the surface of human tissue. Thirty-eight fresh normal tissue samples, 33 paraffin-embedded normal tissues samples, 43 paraffin-embedded epithelial tumor samples including tumors of the colon, stomach, breast, lung, prostate, bladder and pancreas and 23 fresh epithelial tumor samples were examined and graded (Table 3). These results indicate that the generated rabbit sera displays weak binding in the majority of normal samples, similarly observed for the Lewis Y specific BR55-2 Mab. As expected, strong binding was observed in the majority of tumors examined. These data further suggest that the generated sera is minimally binding to normal tissues while displaying strong binding to tumor tissues that over express histo-blood group related carbohydrates. This data suggests that even though this sera reacts with common structural features of MCP and LeY the presentation of related carbohydrate forms on tissues is affected by the carrier molecules to which they are attached as previously suggested (12) or the density of expressed carbohydrate is low.

Carbohydrate modification affects sera reactivity.

The ability of the peptides to mimic carbohydrate fragments or subunits on the cell surface is further observed from consideration of treating cells with neuraminidase and then letting the sera react with the cell (Figure 4). Treating SKBR3 cells with neuraminidase marginally decreased the anti-LeY BR55-2 antibody binding to the cells, while marginally increasing reactivity with the peptide antisera (Figure 4a). This result

indicates that some of the carbohydrates are sialylated (i.e.STn) on the SKBR3 line and their removal may affect the conformational properties of some carbohydrates, exposing new epitopes recognized by the antisera. Treatment of the WM793 human melanoma line with neuraminidase significantly decreased ME361 recognition of these cells, consistent with the recognition of sialyl subunits on the GD2/GD3 antigen (Fig. 4b) Significant increases in the mean fluorescence was observed for antisera binding to WM793 cells. The increase of binding of the anti-sera is interpreted as exposing otherwise encryptic epitopes on the cell surface after sialic acid removal. The core structure for GD2 is GalNAcβ1--->4Gal[3<---2αNeuNAc8<---2aNeuNAc]β1---->4Glcβ1---->1Cer and for GD3 (NeuNAcα2--->8NeuNAcα2---->3Galβ1---->4Glcβ1--->1Cer). Presumably, elimination of sialic acid results in exposure of GalNAcβ1-4Gal units associated with GD2 and Galβ1-4Glc units associated with GD3. Representative synthetic probes of these subunits are highly reactive with the antisera (Fig. 3a). This data further suggests that carbohydrates on the surface of tumor cells are seen by the anti-peptide sera.

To further determine the immunochemical characterization of cell surface carbohydrates, we have performed immunoprecipitation of tumor cell lysates before and after treatment with tunicamycin (Figure 5). LeY epitopes are found to be expressed on MUC-1 mucins, lower m.w. glycoproteins and glycolipids, as well as higher m.w. proteins like CEA and LAMP-1 (29-31). BR55-2 immunoprecipitates (IP) neoproteins in the range >200,000 < 43,000 found on SKBR3 cells (Figure 5a). This profile is similar to that observed for anti-LeY antibodies B1 and B3 (29), and BR96 (30). IP with NMS indicates no reactivity within this molecular weight range (Figure 5a, panel A, second lane). Treatment of SKBR3 cells with tunicamycin for 2 hrs decreases neoglycoprotein reactivity with BR55-2, verifying the carbohydrate recognition of this antibody (Figure 5a, panel B, first lane). IP of SKBR3 cells with the anti-peptide sera indicates that the P1 and P2 anti-sera display an IP profile similar to that of BR55-2 (figure 5b). Strong reactive bands for P1 and P2 are in the range around 47 to 89KD, with weaker bands between 117 KD and 89 KD. These IP bands correspond to LAMP-1 reactive KDs originally identified with the anti-LeY monoclonal antibody BR96 (30). We found that treatment of cells with tunicamycin for 2 hrs. decreases carbohydrate expression of neoglycoproteins reactive with the anti-sera (Figure 5c). However, unlike BR55-2 reactivity, the respective sera is reactive with suspected glycoproteins around 47KD after 2 hrs. Bands around the 47KD

region are identified by BR55-2 prior to neuraminidase treatment. It is possible that our sera is reacting with human breast carcinoma antigen BA46 since immunoprecipitation profiles indicate a protein at 46KD (32). This protein contains an RGD tract in its EGF-like domain. We observed however that antisera directed toward the YYRGD motif does not immunopercipitate any of the glycoproteins in the cell lysates pre and post treatment. This data further suggests that the anti-sera is reactive with carbohydrate epitopes on the cell surface which is similar to that observed for anti-LeY monoclonal antibodies.

Tumor cell cytotoxicity

It has been suggested that anti-carbohydrate antibodies might mediate complement dependent cytotoxicity (CDC) better than cytotoxicity associated with various effector cells (33). Subsequently, we have initially examined CDC mediation of various sera raised either to peptides or against the multivalent LeY-PAA form. This form has been shown to be immunogenic in mice when adsorbed onto bacteria (Salmonella minnesota) (34). We examined the ability of the sera to mediate complement dependent cytotoxicity (CDC) of the SKBR3 and MCF7 human breast adenocarcinoma cell lines, the ovarian tumor cell line OVAR-3 and the human melanoma lines WM793 and SKMEL-28 (Table 4) compared to that of the LeY-PAA generated sera. Positive control antibodies were BR55-2 which mediates CDC of the adenocarcinoma lines, and ME361 which mediates killing of WM793. Negative controls were pre-immune sera, an irrelevant peptide immunogen (C1) and two peptides (G1 and G2) isolated from phage display that are reactive with ME361 (our unpublished observation; to be presented elsewhere).

In Table 4, peptides P1, and P2 both showed an ability to mediate CDC of the LeY expressing human breast lines SKBR3 and the human ovarian line OVAR-3 similar to the positive control BR55-2 MAb. Sera raised against P2 showed diminished CDC activity for MCF7. P2 mediated CDC of the human melanoma lines close to non-specific values using control sera (C1), while P1 displayed moderate CDC activity. The differential CDC activity for the adenocarcinoma and melanoma cells by P1 and P2 antisera are also reflected in the displayed CDC activity mediated by antisera to the G1 and G2 peptides (to be discussed elsewhere). While only several LeY expressing lines are shown in table 4, these data indicate that the functional response can be specific for carbohydrates highly expressed on human tumors. The P2 reactive sera displays a clear preference for the adenocarcinoma cells, while P1 reacts slightly more with the

ganglioside expressing cells. These data also suggest that despite the broad specificity of the sera for carbohydrate constituents by ELISA, the respective sera recognize ubiquitous carbohydrate subunits differently when expressed on cells. These data subsequently indicate that sera generated to carbohydrate mimicking peptides have the potential to recognize important tumor associated antigens with a high degree of specificity.

Discussion

It has been hypothesized that peptides may substitute for carbohydrates in reactions with carbohydrate-specific molecules. The basis of peptide binding (antigenic mimicry) to anti-carbohydrate antibodies differs between antibodies, determined mainly by the antibody combining site (35). It is now evident that one antibody does not necessarily bind to a single antigen, but it may recognize antigens complementary to its combining site even though the chemical components of the antigen may be very different, such as carbohydrates and peptides. The chemical nature of the mimicry between a carbohydrate and mimicking peptide is not completely understood; however recent evidence has shown that aromatic-aromatic and hydrophobic interactions are critical chemical forces between carbohydrate mimicking peptides and their antibody-binding site (19, 20, 22, 23). Immunization with simple synthetic LeY-conjugates only result in sera and MAbs reactive with the immunizing antigen (14). In contrast, immunization with LeY expressing cell lines yield MAbs that react with both synthetic LeY and natural (mucins and cells). One difference between these two antigen sources is the distribution of LeY epitopes on the carrier - in the neoglycoprotein conjugates single LeY structures are substituted over the surface of the carrier protein, whereas in cells, LeY structures are substituted on a variety of carriers (e.g. mucins or glycolipids) on some of which the epitope density is probably quite high. These results indicate that better ways to synthesize LeY immunogens that are reflective of naturally expressed LeY structures (15) or alternative ways to induce immune responses cross-reactive with native LeY are needed. One alternative is the use of peptides as surrogate antigens. Here, we have shown that peptide motifs containing aromatic residues can effectively mimic the antigenicity and immunogenicity of carbohydrates associated with those expressed on the surface of adenocarcinomas.

We have recently determined the molecular recognition properties of the tetrasaccharide LeY antigen to anti-LeY antibodies (28) and have shown that the LeY tetrasaccharide structure is similar to the core structure of MCP (23), suggesting that antibodies reactive with aromatic containing peptides might also react with histo-blood-group related carbohydrate subunits. ELISA screening with an anti-LeY antibody (BR55-2) which recognizes a biologically active conformation of LeY on the tumor cell surface suggests that the YRY and WRY motifs better reflect a LeY like topographical feature which is complementary to the BR55-2 combining site, with diminished reactivity against the YPY motif (figure 1). This result suggests that the substitution of proline diminishes the functional antigenic mimicry of the aromatic-aromatic motif for BR55-2 binding. This is especially apparent in that the anti-LeY antibody 15-6A exhibits very little binding to the YPY motif. Of interest is the lack of reactivity of BR55-2 or 15-6A for K61223 which is a derivatized form of a peptide motif isolated from phage display with another anti-LeY antibody B3 (27). We have recently put forth a structural explanation for the lack of reactivity of BR55-2 with the APWLY peptide (36). These data further emphasize that antibodies discriminate peptides far better than carbohydrate forms (35). In contrast, we do observe that ME361 reacts with the YYRYD motif, indicating that the sialic acid moiety which ME361 binds to is adequatey mimicked by the motif.

To test the immunological mimicry of the aromatic-aromatic containing motifs, mice were immunized with proteosome conjugates or as MAPs. The utility of MAP peptides is in their apparent advantage as immunogens (37, 38). MAPS have proved to retain all the immunological properties of an intact anti-id for example upon which the peptide was based (37, 38), and was found to be qualitatively similar and quantitatively superior to the linear monomeric 15mer anti-Id derived peptide (38) when MAPS contain a T cell epitope. ELISA reactivities against synthetic probes indicate that the respective anti-peptide sera recognize a broad range of subunits associated with the blood-group related antigens, and Lewis extended structures, while BR55-2 reacts specifically with the LeY probe (Figures 2 and 3). We have attributed the BR55-2 specificity to its interaction with the GlcNAc residue on the tetrasaccharide core, as well as the number of hexose units comprising the LeY extended structure (28). Superpositioning of MCP and LeY structures emphasize the spatially conserved moieties involving Fuca1-3GlcNAc on LeY (23). While a distinction is observed between IgG sera reactivity for Fuca1-3GlcNAc (Figure 3a), the results indicate very

little discrimination in carbohydrate subunits. Subsequently, while BR55-2 specifically recognizes the YRY motif, the YPY motif must induce a subset of antibodies whose combining sites are complementary to both the immunizing peptide and the respective carbohydrate subunits. In comparison with the MAP peptides, we found the predominate Ig fraction to be of IgM isotype as observed with other MAPs] (39). IgM antibodies are clearly more "sticky" for carbohydrate moieties. Nevertheless, we observe a better degree of specificity for the LeY structure over other Lewis antigen forms. These results suggest that multivalent presentation of the peptide epitopes improves a motif specificity's, possibly mediated by avidity, similar to previous observations (37, 38).

Reactivity of sera generated against the YRYD motif with human normal and tumor specimens indicate weak reactivity with normal tissues (Table 3). Reactivity of a common carbohydrate epitope with different antibodies or ligands is highly dependent on the type of carrier glycosylceramide or carrier O-linked peptide (12) which can effectively restrict cross-reactivity with otherwise related carbohydrates expressed on normal tissues. It is very possible that antibodies may not cross-react with tumor cells expressing carbohydrates, while reacting with carbohydrate probes in ELISA. Published reports on polyclonal sera raised to GM2, sTn and LeY preparations, all react to these antigens in ELISA but do not react with native forms on the tumor cell surface (10, 13, 14). Furthermore, reactivity of a common carbohydrate epitope with different antibodies or ligands is highly dependent on the type of carrier glycosylceramide or carrier O-linked peptide (12) which can effectively restrict cross-reactivity with otherwise related carbohydrates expressed on normal tissues. The tumor association of a trisaccharide epitope similar to the histo-blood group antigens, galactose beta1->3 N-acetyl glucosamine beta1-->3 galactose, indicates that the epitope is highly restricted to adenocarcinomas as assessed by rabbit sera made to the trisaccharide coupled to BSA (40). Our immunization studies also suggest that if sera is reacting with normal tissues in mice and rabbits, there is no apparent adverse reactions.

Further evidence that our sera is reacting with carbohydrates on the cell surface comes from treatment of cells with neuraminidase (Figure 4). Treatment appears to expose carbohydrate subunits representative of the synthetic probes in figure 3 that display reactivity with the antisera. The reactivity of the anti-peptide sera with LeY is further observed from consideration of the IP profiles in figure 5. These results indicate

reactivity with neoglycoproteins can be mediated by treatment with tunicamycin, further supporting the notion that the sera is reactive with carbohydrate moieties expressed on the cell surface. It is possible that our sera is reacting with human breast carcinoma antigen BA46 since immunoprecipitation profiles indicate a protein at 46KD (32). Antibodies to this site have been used to mediate tumor regression in mice (32). As shown sera to the YYRGD motif does not IP this band. In related studies, we have tested sera for reactivity against fibrinogen, fibronectin, vitronectin and von willebrane factor since the RYD motif is similar to the RGD motif on the aforementioned molecules. We showed previously that a RYD peptide only interacts with the platelet protein GPIIb-IIIa (41). We found no reactivity with these molecules in ELISA assays (data not shown). This result is in keeping with other observations that the RGD motif on the aforementioned molecules display different conformations and do not necessarily induce anti-RGD antibodies that cross-react with all RGD motifs (42). These latter observations are key elements in developing peptides of the aforementioned type as potential immunotherapeutic agents.

Our CDC results (Table 4) indicate that the sera is specific for LeY expressing cells. This might be the result of increased avidity for LeY. The data also suggests that multivalency is of importance in generating responses to natively expressed LeY on tumor cells and that peptides might do a better job of inducing sera that targets LeY expressing cells. It is well known that the binding of an antibody to a cell surface is governed by the density of antigen expressed. This is particularly true for IgM antibodies. The pentameric and hexameric nature of IgM facilitates binding to clusters of antigens changing from a "planar" to "staple" conformation when it binds to clustered epitopes. The "staple" conformation facilitates complement fixation and complement-mediated lysis. IgM antibodies induced in patients may not bind to normal cells (lymphocytes, granulocytes or monocytes) because the antigens on the surface of normal cells are expressed in low concentrations or are sparsely distributed (6). In the absence of sufficient density of epitopes, IgM may not bind to bring about the staple conformation. It is noteworthy that patients who developed high titers of anti-siaylated Lewis (sLe) antigen IgM showed no evidence of hematologic toxicity (hemolysis, anuria or granulocytopenia) (6).

In summary, these studies indicate that carbohydrate structures can be mimicked by peptides and suggests that appropriately constructed peptides may indeed be able to augment immunogenicity against

carbohydrate antigens. Peptide mimics may be designed as polymeric peptides mimicking more complex carbohydrates, or polyvalent vaccines may be produced using heteropolymers of mimicking peptides. Mimicking peptides represent a new and very promising tool to overcome T-cell independence and to increase efficiency of the immune response to carbohydrates. Subsequently, peptides that mimic tumor associated carbohydrates would be of importance as novel agents for adjuvant therapy.

Materials and Methods

Preparation of Peptide immunogens.

Several peptides were synthesized, repeating putative centralized motifs suggested to mimic carbohydrate forms and correspond to GGIYYPYDIYYPYDIYYPYD (K61105), GGIYWRYDIYWRYDIYWRYD (K1106), GGYYRYDIYYRYDIYYRYD (k61107), GGGAPWLYGAPWLYGAPWLY (K61223), GGAPWLYGGAPWLYAPWLY (K61108). Other peptides were synthesized as variants that include GGAGRWVFSAPGVRSIL (K6111), GGGWPYLRFSPWVSPLG (K61110), GGARVSFWRYSSFAPTY (K61109). Peptides were synthesized with the addition of a tripeptide YGG spacer, and a cysteine at the amino terminus conjugated to a lauroyl group (22, 23) (Bio-Synthesis, Lewisville Texas) or as Multiple Antigen Peptides (MAPs) (Research Genetics, Huntsville Alabama). For the proteosome conjugates, the meningococcal outer membrane proteins or proteosomes were prepared and complexed to the Lauroyl-C-YGG-Peptides as described by Lowell et al. (43, 44) in a 1:1 ratio, combining the components in the presence of detergent. The detergent was removed by extensive dialysis (43, 44). The lauroyl group allows for hydrophobic complexing of the peptide to the proteosomes while the cysteine at the N terminus appears essential for immunogenicity apparently cross-linking multiple epitopes. MAP peptides were made by Fmoc synthesis on polylysine groups resulting in the presentation of 8 peptide clusters (38, 45). Peptides were 95-99% pure as assessed from HPLC preparation.

Preparation of Antibodies Against Carbohydrate-Mimicking Peptides

For generation of polyclonal sera Balb/c mice (n=4 per group) 4-6 weeks of age, were immunized i.p. on a weekly basis for 3 weeks with 50ug of peptide-proteosome complex as described (22). Other groups of mice were administered with 50 ug of the respective MAPs and 20ug of QS-21 adjuvant (Aquila

Pharmaceuticals, Worcester MA), at intervals of 2 weeks for 6 weeks. Sera was collected within 7 and 14 days after the last immunization and stored at -20^{O}C.

ELISA assays

Solid phase ELISA was performed to assess the binding activity of anti-carbohydrate monoclonal antibodies and ploYclonal anti-peptide sera to MAPs or a variety of carbohydrate synthetic probes incorporated into a polyacrylamide (PAA) matrix (Glycotech, Rockville Md.). For the peptide ELISAs, MAP peptides were coated on Immulon 2 plates (2ug/well) and reacted with 0.2 ug of the anti-LeY monoclonal antibodies BR55-2 and 15.6, developed against MCF-7 cells (46, 47) or the anti-GD2/GD3 antibody ME361 (48). For peptide inhibition, plates were coated overnight with LeY-PAA at 0.1 ug/well. The MAbs (0.1 ug) or sera were admixed with varying concentrations of MAP peptides for 15 min on ice, and then allowed to react with LeY coated plates. For sera evaluation of anti-carbohydrate activity, Immulon 2 plates were coated with a variety of carbohydrate probes that included Fucα1-4GlcNAc, LeY, Galβ1-3Gal, Galβ1-3GalNAc, Sialyl-Lea, Lea, Sialyl-Lex, Lex, Lex-pentasaccharide and Leb-hexasaccharide. Plates were coated with 2ug/well of the respective probes overnight at 4^{O}C and blocked (23). Serial dilutions of the respective anti-sera was added and resolved with 100 μl / well of 1 : 10000 anti-Mouse isotype matched-HRP (Sigma) diluted in blocking buffer, incubated at 37^{O}C for 1hr. OD_{450nm} was read for all ELISAs using a Dynatech MR5000 ELISA reader. All results were calculated from triplicate measurements.

Flow cytometry

Representative human LeY expressing cell lines include the breast cancer lines SKBR3, SKBR5, MCF7, OVAR-3 (ATCC (Rockville, MD). Control cell lines include the human non-LeY expressing cell line HS578 Bst (normal breast cell line, ATCC), and the human melanoma line WM793 (gift from D. Herlyn, Wistar Institute) , SKMEL- 28 (ATCC) and NIH3T3 murine fibroblast. For the preparation of cells, 10ml of FACS buffer was added and the cells were washed, scrapped and transferred to 15 ml. centrifuge tubes. Viable cells were counted by trypan blue. Cells were diluted to 2 X10^{6}/ml and 100ul used for each sample. Primary sera (10ul) was added to the sample tubes and incubated on ice for 30 min. washed twice with 1 ml FACS buffer and centrifuged for 5 minutes at 1500 rpm. 10 ul of FITC Ab (goat anti-mouse IgG or IgM FITC labeled (Sigma) diluted 1:20 with PBS) was added to the sample and incubated on ice for 30 min. and

again washed twice with FACS buffer. Cells were fixed using 2% paraformaldelhyde, followed by FACS measurement on a Becton Dickinson flow cytometer FACScan (Becton Dickinson, Los Angeles CA). Cells were also treated with neuraminidase to remove sialic acid residues on carbohydrates expressed on the cell surface. In these assays $4X10^7$ cells/ml were treated with Neuramindase (0.1 unit/ml in RPMI 1640) on ice for 2 hours. Cells were spun down and ice cold medium, containing 0.1 mg/ml Fetuin, was added and incubated on ice for 15min. Cells were washed with cold medium and reacted with antibody as described above.

Identification of tumor surface expressed LeY

Neoglycoproteins expressing LeY were detected by immunoprecipitation. SKBR3 cells were plated at $3X10^6$/ flask (T-75 flask) 24 hours before labeling. Medium was removed and replaced with 4 ml of Methionine and Cysteine free Dulbecco's modified Eagle's medium containing 10% fetal bovin serum and incubated at 37°C for 60 minutes. Cells were then labeled biosynthetically with 200uCi of ^{35}S Methionine and ^{35}S Cysteine for 4 hrs. To one group of cells, 5ml of complete media was added and incubated for 2 hrs at 37°C with the glycosylation inhibitor Tunicamycin (10µg/ml). Labeled cells were harvested and washed with PBS. Immunoprecipitation was performed as previously described (49) with some modification. Cells were extracted with 0.02 mol/L Tris-HCL buffer, pH 7.2, containing 1% Triton X-100 (Sigma Chemical Co, St. Louis MO) for 60 min at 4°C and extracts were cleared of particulate debris by sedimentation at 10,000g. Five hundred microliter aliquots of the supernatants were incubated with 4 uL of normal mouse serum for 15 minutes at 4°C, followed by an incubation with 100 uL of a 10% suspension of fixed Staphylococci (Pansorbin; Calbiochem, San Diego, CA) for 30 min at 4°C. After sedimentation at 12,000g to remove the Staphylococci, 15 µg of antibody or 6µl of sera was added to the cleared supernatant and the incubations were continued at 4°C for 1 hour. Immune complexes were collected by adding 50µl Protein A and 50µl Protein G beads. After centrifugation the pellet was washed three times with DOC-10mM NaCl and DOC-300mMNaCl. The washed pellets were resuspended in 100µl SDS-PAGE sample buffer (10mM tris and 3% SDS pH 6.8 with 60mg/ml DTT), boiled for 4 min, and centrifuged. The supernatant was run on an 8% SDS-polyacrylamide gel.

Distribution of carbohydrate reactivities on human tissues: To further our studies of cross-reactivities, we have initiated screening our sera with human surgical specimens. We performed immunostaining (using an indirect immuno-peroxidase method) of tissue specimens derived from a variety of tumor types and normal tissue following procedures previously described (50). Tissues were obtained from the tissue procurement section of the Hospital of the University of Pennsylvania. Rabbit polyclonal antisera raised against the K61107 peptide-proteosome conjugates were used in this study. Sera was collected after the third immunization. This sera was shown positive for LeY (data not shown). As a control antibody we used BR55-2. Control immunocytochemical experiments were performed by subsitution of primary rabbit sera with normal rabbit serum at the same dilution. Formalin-fixed, paraffin-embedded normal tissue sections representing the major organ systems were selected from surgical diagnostic files. Fresh normal and tumor human tissues (i.e.tonsil,skin,colon, skeletal muscle, and normal tissue surrounding excised tumors) obtained at surgical resection and snap frozen in liquid nitrogen-cooled isopentane, were also examined. Immunocytochemistry was performed following procedures previously described (50).

Fresh tissues were embedded in OCT (Miles) before storage at -70 C. Serial sections from frozen and wax-embedded tissues cut at 5 and 4, respectively are mounted on poly (L-lysine) coated slides, air dried and fixed in acetone (4C, 10 min) before use in immunohistochemistry. Immunostaining is performed using a sensitive three-layer avidin-biotin complex (ABC) method with the rabbit IgG Vectastain Elite ABC (peroxidase) kit as outlined by the manufacturer (Vector Laboratories). Sera to the As a control antibody we used BR55-2. Control immunocytochemical experiments were performed by subsitution of primary rabbit sera with normal rabbit serum at the same dilution. Before application of normal goat serum, fixed and unfixed frozen sections were immersed in PBS, pH 7.4. Similarly, paraffin sections are dewaxed in xylene and hydrated through graded alcohols to water, then subsequently placed in PBS. Primary antisera was diluted to 1:100. After overnight incubation at 4C, sections are rinsed in PBS and incubated for 1 hr at 22C with biotinylated goat anti-rabbit antibody. Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide in methanol for 30 min.; sections were rinsed in PBS and incubated for 30 min with ABC solution. After further washes in PBS, the reaction product was visualized using diaminobenzidine (Sigma)

as chromogen. Sections were counterstained with Harris's hematoxylin, dehydrated with graded alcohol's, cleared in xylene and mounted.

Complement-dependent cell cytotoxicity (CDC)

Sera was tested for its ability to bind to tumor lines and modulate CDC as previously described (14). Briefly, 10 ul of each cell line ($4X10^4$ cells per ml) were added to duplicate wells of microtiter plates and incubated overnight at 37°C. Medium was removed and 20ul of serially diluted sera was added, and incubated for 45 min on ice. Twenty ul of rabbit complement (1:2) was added. After 4 hrs, plates were fixed with methanol for 10 min, rinsed in distilled water, stained with 2% Giemsa stain in phosphate-buffered saline for 25 min, and rinsed. Plates were counted under a light microscope and the percent of cytotoxicity (PC) of a given serum dilution was calculated as follows: Percentage cytoxicity=[1-(number of cells in well treated with serum and complement / number of cells in well treated with medium only)] x 100. Control wells did not contain antisera.

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Figure Legends

Figure 1. Reactivity of putative motifs by ELISA. (A) Reaction of MAbs with respective MAP peptide forms. (B) Inhibition of mAb BR55-2 binding to solid-phase LeY-PAA by soluble MAP peptides. Constant amounts of BR55-2 were incubated with increasing amounts of MAP peptides, and then reaction of free mAb with LeY was measured by ELISA. Data points reflect 50% inhibition at 2 ug/ml of peptide inhibitor as measured by reduction of O.D values in ELISA.

Figure 2. Reaction of anti-peptide sera with LeY and Leb. (A) Reactivity of the IgG portion of peptide-proteosome derived antipeptide sera. (B) Reactivity of IgM portion derived from MAP immunizations. Pre-immune reactivity of IgM with the carbohydrate probes was subtracted from the respective data points in 2B.

Figure 3. Profile of cross-reactivity of anti-peptide sera with carbohydrate probes. (A) IgG anti-peptide reactivity. (B) Carbohydrate-PAA forms reactive with IgM anti-peptide sera. Pre-immune reactivity of the IgM fraction with the carbohydrate probes was subtracted from the respective data points in figure 3B.

Figure 4.. Summary of FACs results for antisera binding to breast and melanoma cells before and after neuraminadase treatment. A. Pre and post treatment of SKBR3 cells. B. Pre and post treatment of WM793 cells. The P1 sera corresponds to the YYPYD motif, and P2 corresponds to the YYRYD motif. Sera in both assays are diluted 1:100.

Figure 5. Immunoprecipitation profiles of SKBR-3 cell lysates with antisera compared with BR55-2. (a) Molecular wt markers from top to bottom are those in figures b and c. 5a.) Panel A first lane is reactivity with BR55-2 while the second lane is normal mouse serum that has been preabsorbed. Panel B is profile after tunicamycin treatment. 5b.) Reactivity of sera before tunicamysin treatment. P1 is sera raised against the YYPYD motif, P2 is sera raised against the YYRYD motif, and P3 is sera raised against the motif YYRGD. 5c.) Reactivity of the respective sera post treatment with tunicamycin (2hrs.)

Table 1. Peptide motifs that mimic carbohydrate structures

Motif	Carbohydrate	Structure
YYPY	Mannose	methyl-α-D-mannopyranoside
WRY	Glucose	α(1-4)glucose
PWLY	Lewis Y	Fucα1→2Galβ1→4(Fucα1→3)GlcNAc
YYRYD	GroupC Polysaccharide	α(2-9)sialic acid

Table 2 Binding of Various Anti-Peptide Sera to Different Cells As Measured by FACS

Cell Lines	Anti P1 (YYPY)	Anti P2 (YYRYD)	Anti 105 MAP	Anti 107 MAP	ME361 (100ug/ml)	BR55-2 (100ug/ml)
SKBR5	60.0	86	ND	ND	3.1	59.0
MCF7	63/144*	54	150	176	5.4	352
SKBR3	240.6	275.6	240	250	3.2	235.6
HS578 Bst (normal breast)	17.8	19.9	18.6	18.4	ND	16.2
SKMEL-28	47.0	33.0	ND	ND	26	13.8
WM793	145.5	42.3	ND	ND	92.1	15.4
NIH3T3 Murine Fibroblasts	20.9	21.8	24.5	21.2	ND	15.3

*Background fluorescence (Mean Fluorescence) associated with non-specific mouse sera is 24.2, and 23.7 for SKBR3, and NIH 3T3 cells, respectively. ME361 is 14.0 and 10.0 for SKBR5 and MCF7. Background for the human melanoma line was on average 24.4. (Final Sera Concentration: 1:50). * final dilution at 1:20.*

Table 3 Summary of Carbohydrate Expression on Human Tissues

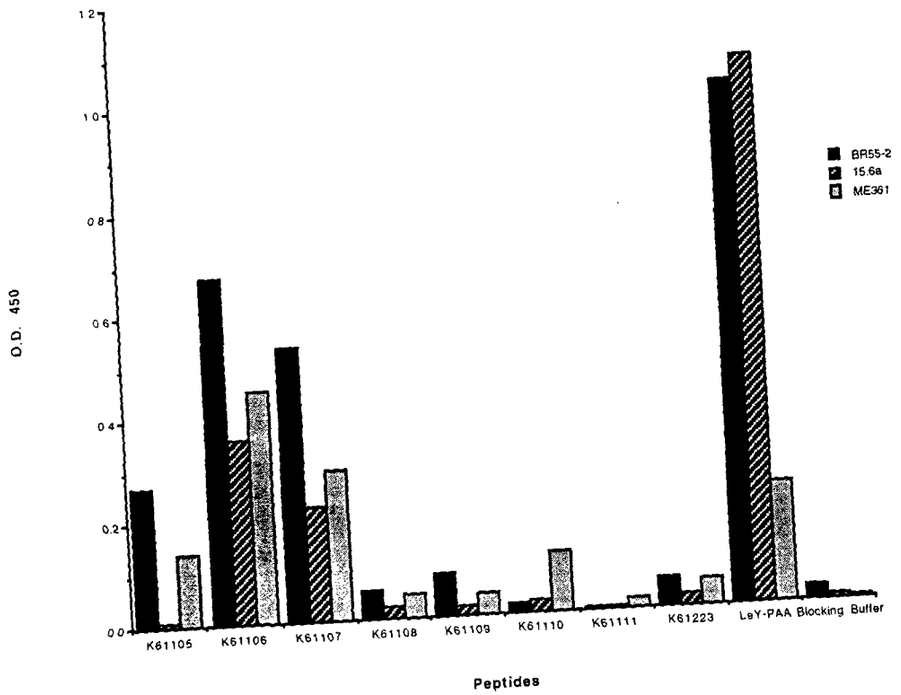
Tissue Type	Total	Sera Reactivity				BR55-2 reactivity			
		+++	++	+	-	+++	++	+	-
Normal tissues									
Stomach	5			1	4				5
Pancreas	7			2	5			2	5
Ovary	20			4	16			2	18
Breast	15			4	11			2	13
Lung	4			2	2			1	3
Heart	2				2				2
Prostate	12			2	10			3	9
Thymus	6			2	4			2	4
Tumors									
Breast	20	20				19	1		
Lung	11	9	2			10	1		
Ovary	20	17	3			17	3		
Pancreas	4	2	2			4			
Bladder	5	4	1			4	1		
Prostate	6	4	2			4	2		

Carbohydrate expression was determined by the avidin-biotin-immunoperoxidase method and scored according to staining intensity and abundance of immunostaining:+++, strong, ++ moderate, + weak, - negative. The numbers under Total refer to numbers of individual samples from different individuals that were tested. The numbers in the body correspond to the number of samples that fall into specific categories of reactivity.

Table 4. Summary of Complement Dependent Cytotoxicity Results

Tumor	C1	P1	P2	G1	G2	LeY-PAA	ME361	BR55-2
SKMEl-28	3	32	10	75	87	4	35 (50µg)	3 (100µg)
SKBR3	6	90	86	10	13	20	10 (100µg)	80 (100µg)
MCF-7	3	66	29	20	15	26	5 (50µg)	75 (100µg)
WM793	5	28	9	90	90	2	63 (30µg)	1 (100µg)
OVAR-3	5	89	86	9	11	25	6(50µg)	80 (100µg)

Values are averaged percent cytotoxicity. Final dilutions are 1:15 for sera. Monoclonal antibody ME361 and BR55-2 concentrations are per ml.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0.6
0.4
0.2
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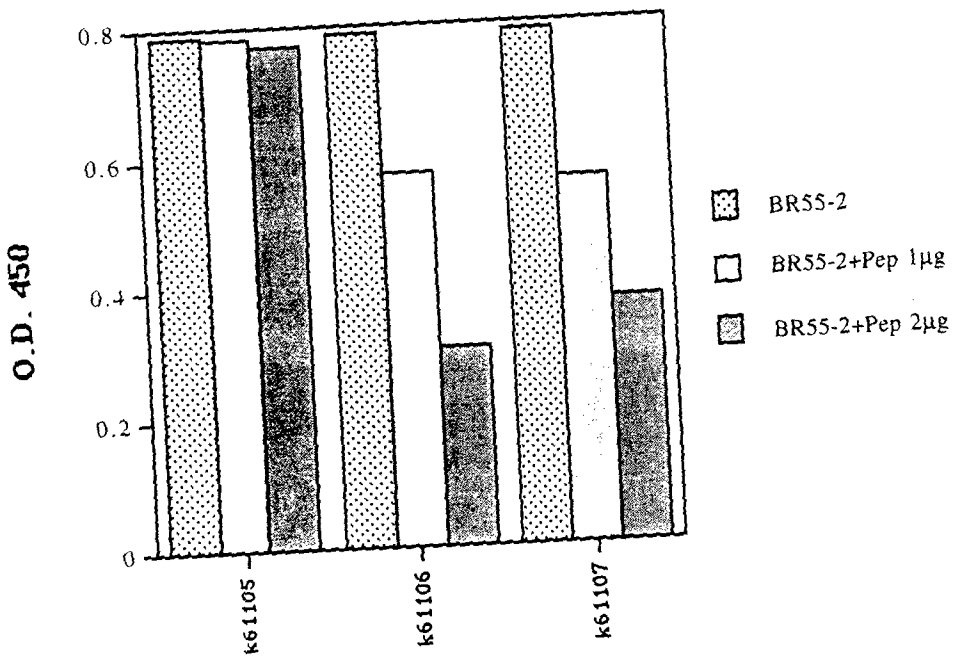
O.D. 450
BR55-2
15.6a
ME361
K61105
K61106
K61107
K61108
K61109
K61110
K61111
K61223
LeY-PAA
Blocking Buffer
Peptides

Figure1a.


O.D. 450
0.8
0.6
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0.2
0
k61105
k61106
k61107
Peptide Inhibitor
BR55-2
BR55-2+Pep 1μg
BR55-2+Pep 2μg

Figure 1b.

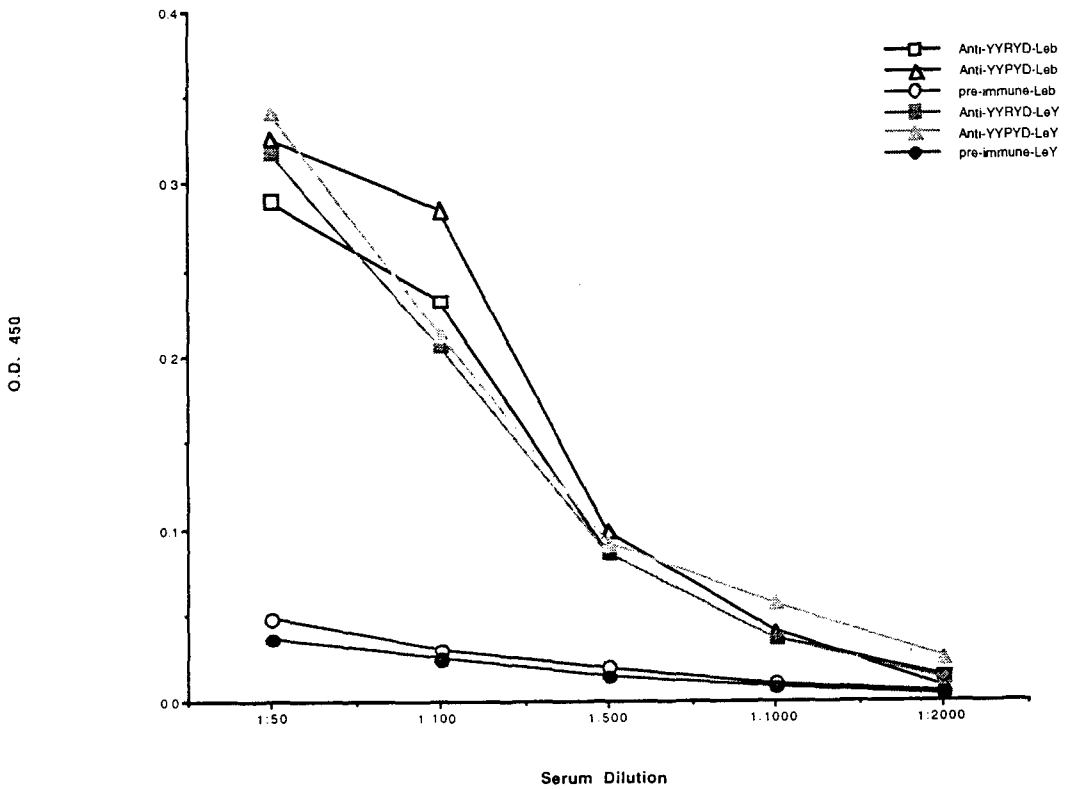

0.4
0.3
0.2
0.1
0.0
O.D. 450
1:50
1:100
1:500
1:1000
1:2000
Serum Dilution
Anti-YYRYD-Leb
Anti-YYPYD-Leb
pre-immune-Leb
Anti-YYRYD-LeY
Anti-YYPYD-LeY
pre-immune-LeY

Figure 2a

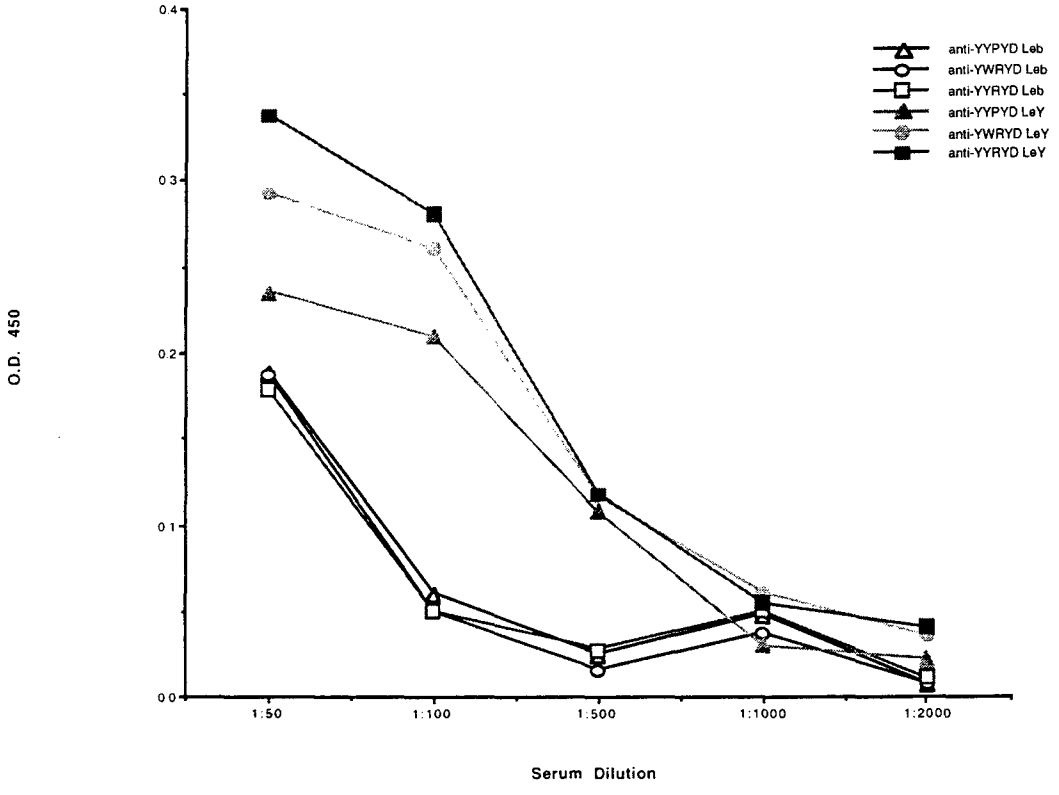

0.4
0.3
0.2
0.1
0.0
O.D. 450
1:50
1:100
1:500
1:1000
1:2000
Serum Dilution
anti-YYPYD Leb
anti-YWRYD Leb
anti-YYRYD Leb
anti-YYPYD LeY
anti-YWRYD LeY
anti-YYRYD LeY

Figure 2b

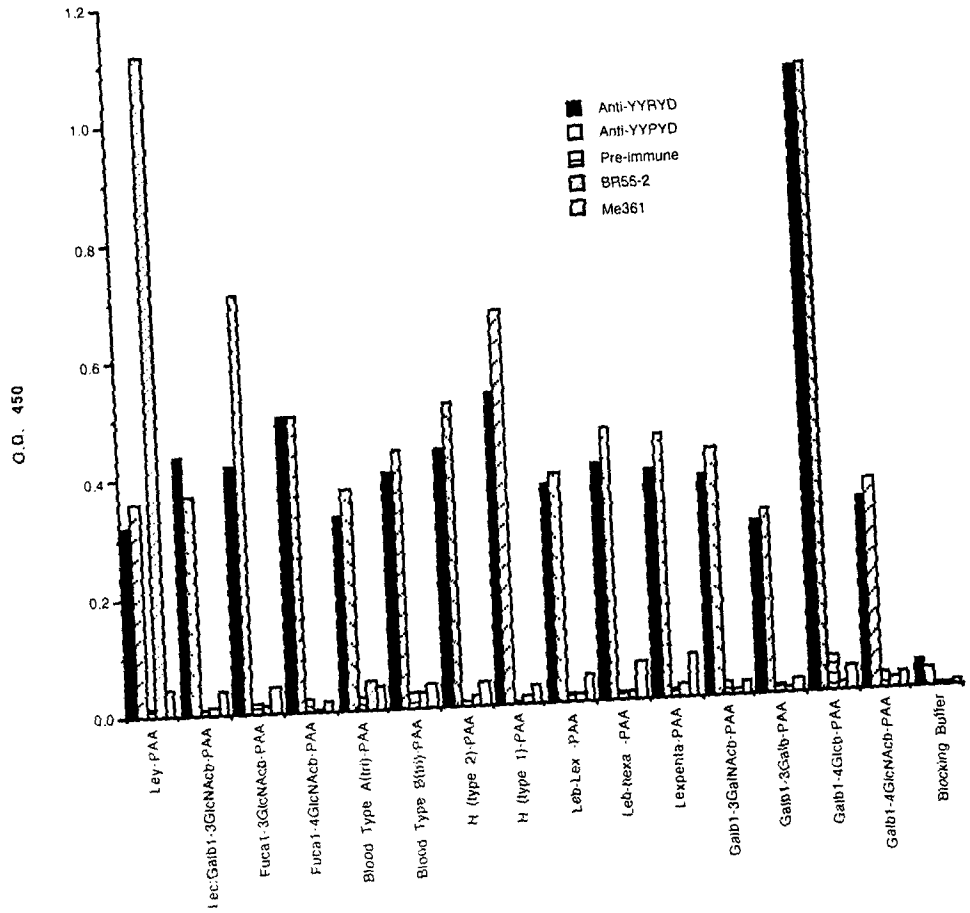

O.D. 450
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Anti-YYRYD
Anti-YYPYD
Pre-immune
BR55-2
Me361
Ley-PAA
Galb1-3GlcNAcb-PAA
Fuca1-3GlcNAcb-PAA
Fuca1-4GlcNAcb-PAA
Blood Type A(tri)-PAA
Blood Type B(tri)-PAA
H (type 2)-PAA
H (type 1)-PAA
Leb-Lex -PAA
Leb-Hexa -PAA
Lexpenta-PAA
Galb1-3GalNAcb-PAA
Galb1-3Galb-PAA
Galb1-4Glcb-PAA
Galb1-4GlcNAcb-PAA
Blocking Buffer

Figure 3a

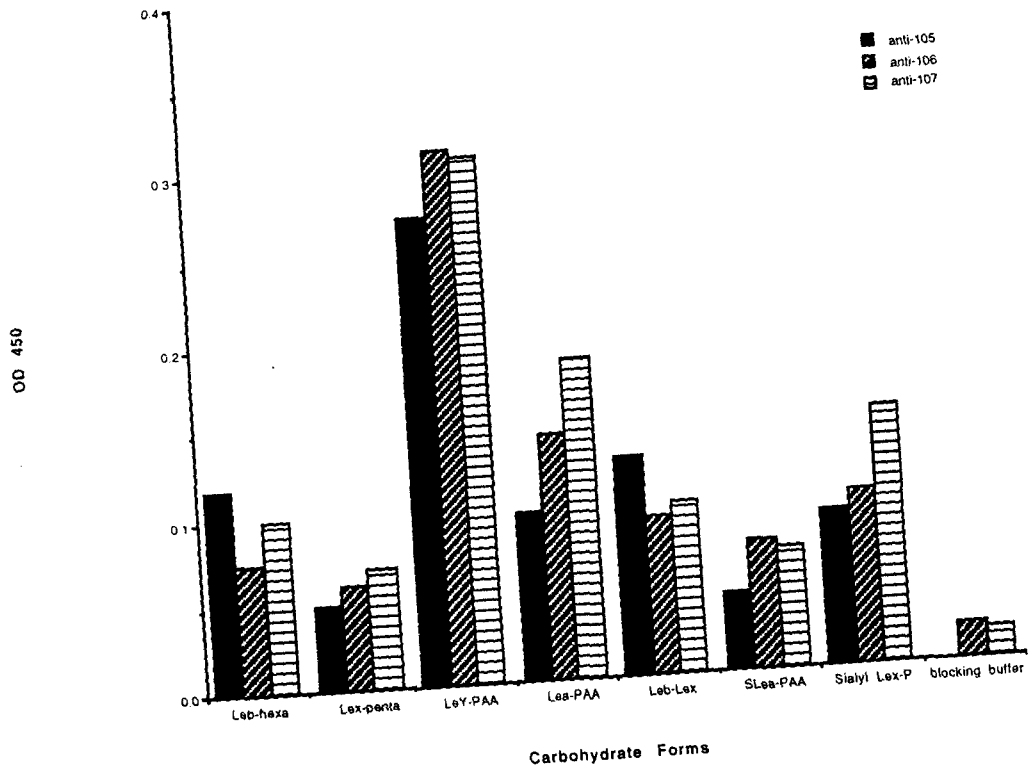

0.4
0.3
0.2
0.1
0.0
OD 450
anti-105
anti-106
anti-107
Leb-hexa
Lex-penta
LeY-PAA
Lea-PAA
Leb-Lex
SLea-PAA
Sialyl Lex-P
blocking buffer
Carbohydrate Forms

Figure3b

Figure 4a.

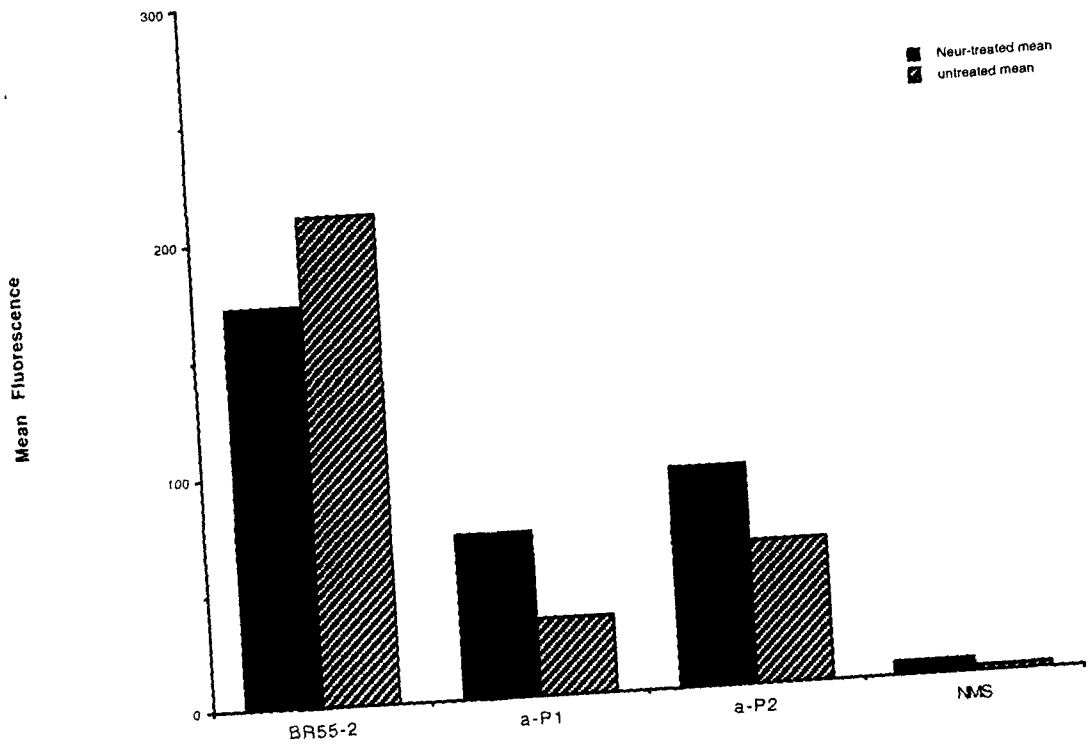
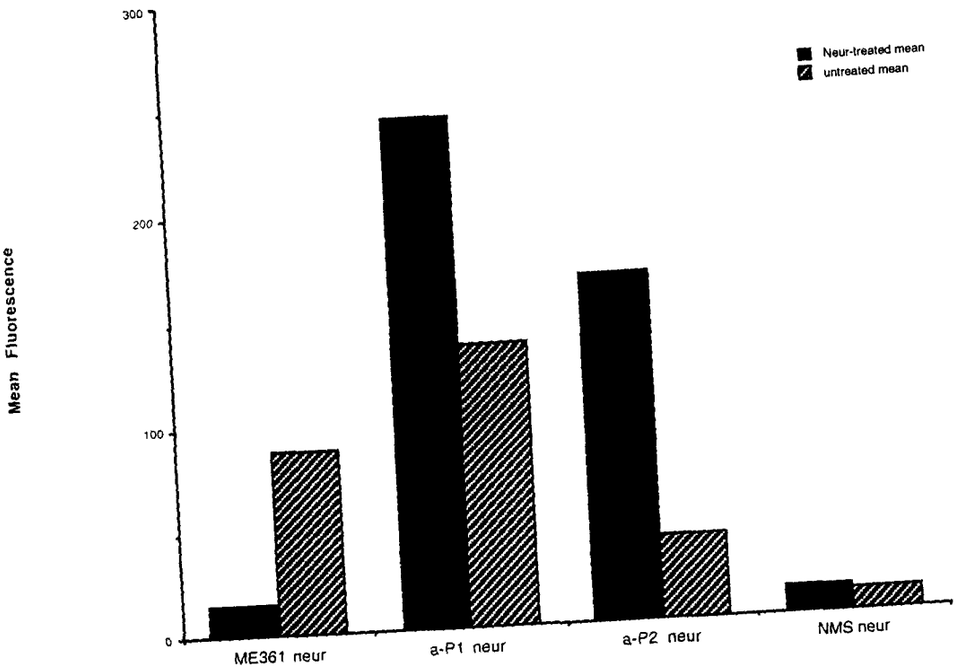

300
200
100
0
Mean Fluorescence
Neur-treated mean
untreated mean
BR55-2
a-P1
a-P2
NMS

Figure 4b


300
200
100
0
Mean Fluorescence
Neur-treated mean
untreated mean
ME361 neur
a-P1 neur
a-P2 neur
NMS neur

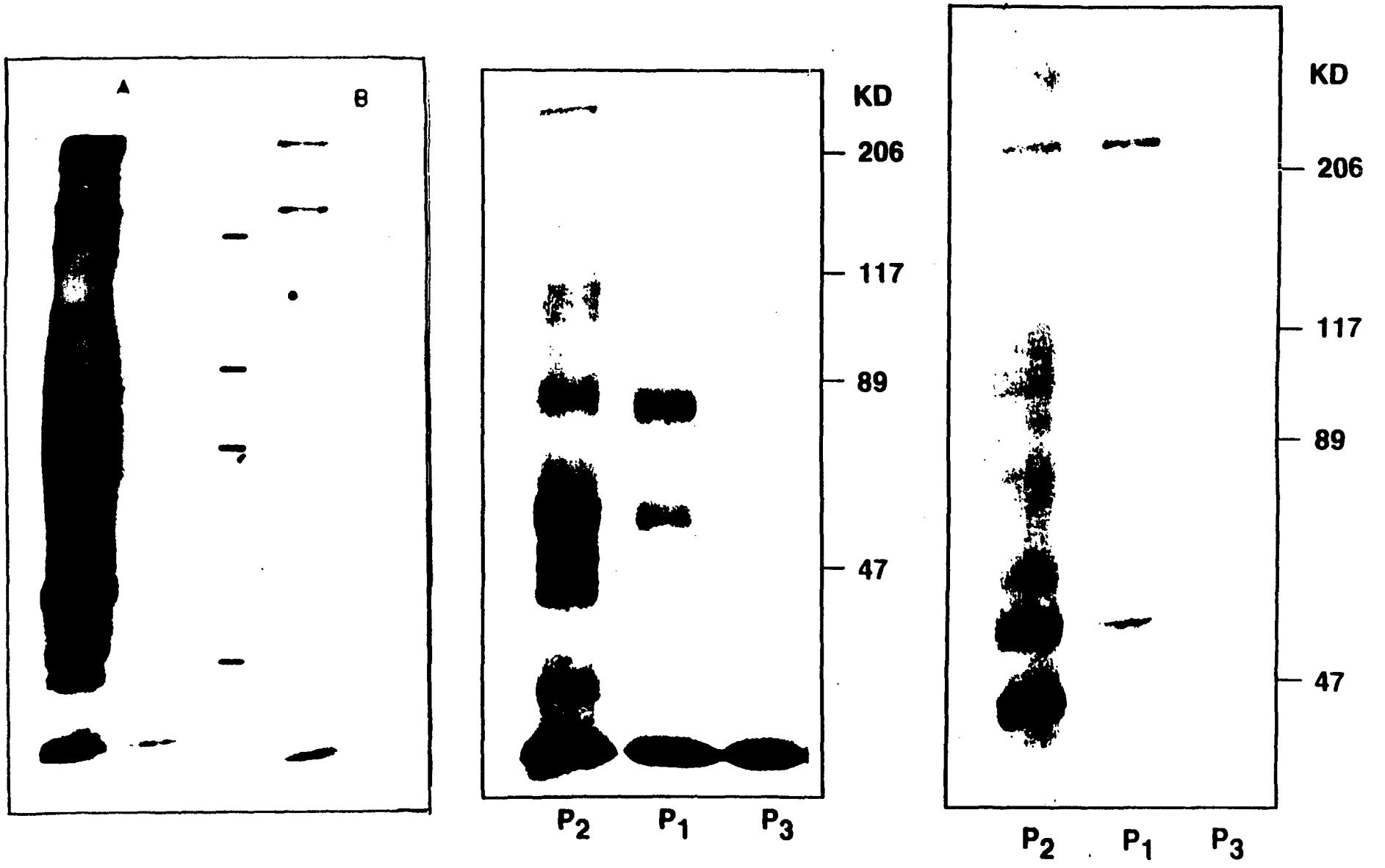


Figure 5

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I would like the following material limited or held confidential: Table 2, Table 3, Table 4, Table 5, Figure 2, Figure 3, Figure 4, and Figure 5. In addition, manuscript #4 is only submitted and should not be distributed in an unlimited fashion.

Thank You

Thomas Kiebr-Emmons

Rec'd
7/19/2000

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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